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Characterize a Broad Spectrum of p53 Mutations Associated
with Breast Cancer

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13. ABSTRACT (Maximum 200 Words)
The p53 tumor suppressor protein is an inducible, sequence-specific transcription factor capable of modulating the expression of more than 100 genes in human cells to coordinate different cellular responses to stress. The p53 pathway is altered in nearly all human cancers and p53 mutant proteins with amino acid changes in the DNA binding domain are aberrantly expressed in nearly 50% of tumors. We developed a system in the yeast *Saccharomyces cerevisiae* that addresses p53 transactivation capacity from 26 different p53 *Response Elements* (REs) in a constant chromatin structure and that provides for detailed functional analysis of p53 mutant alleles. We applied this method in trying to elucidate the mechanisms that regulate differential transactivation by p53 and determined the functional fingerprints of many p53 mutants including a group of novel alleles that were reported in familial breast cancer. Interestingly, we observed that among ~1300 different amino acid changes detected in tumors, many do not result simply in loss of function but instead alter the ability to transactivate from the various target sequences. Our results suggest that the actual functional status of p53 alleles expressed in tumor cells, not simply whether or not there is a mutation, may correlate with clinical outcome. The yeast functional assay offers a practical means to develop a *p53 mutant functionality database* that is likely to become valuable in predicting tumor aggressiveness and responsiveness to therapy.

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INTRODUCTION

Multiple perturbations of the complex network of signaling pathways that define the

precise role of a cell in its tissue microenvironment and that regulate stress responses are

accumulated through genetic and epigenetic changes during processes of transformation

that lead to cancer (1). The tumor suppressor gene p53 plays a major role in cellular

response to various environmental stresses (2). The p53 protein is a sequence-specific

transcription factor that can lead to transactivation and repression of over 100 genes,

many of which are involved in apoptotic or cell cycle arrest responses. The induction of

p53 responses can lead to different biological effects according to the cell type or the

activating stimuli. For example, temporary G1 or G2 cell cycle arrest, premature

senescence and programmed cell death can all be induced (or maintained) by p53 (3).

The mechanisms of how the choice between these pathways is brought about are still

elusive, although several possibilities have been proposed. Differential regulation of

downstream genes, both in terms of the extent and the kinetics of transactivation and

repression, is likely to be an important factor for dictating the specificity of p53

biological responses. Functional alterations in the p53 pathway likely occur in nearly all

human cancers. In almost half the human malignancies, there is a mutation in the p53

gene itself (4, 5). Interestingly, ~80% of p53 mutations are missense changes that lead to

single amino acid substitutions, a feature that distinguishes p53 from other tumor

suppressor genes (*e.g., APC, NF1, BRCA1*) (6). The incidence of p53 mutations and the

types of mutations can vary among tumors in different tissues or populations (4) and the

exposure to some environmental agents has been associated with the features of p53

mutation spectra in tumors (7). About 1300 different amino acid changes at p53 have

been reported in tumors and a significant fraction of these do not lead to loss of function,

but can retain partial functions or exhibit altered biological activities. Several recent

reports support the view that certain *p53* mutant alleles can retain partial function. Some

mutations are associated with partial transactivation and are capable of inducing G1

arrest, but not apoptosis (8). Furthermore, a recent analysis of 77 different p53 mutant

proteins from tumors revealed that >15% could still activate a yeast promoter containing

a *p21* RE, but not the *Bax* or *PIG3* RE (9), which presumably is important in tumor

development. A few *p53* mutant alleles appear to have acquired functions that may

provide a selective advantage to tumor cells, such as the up-regulation of growth

promoting genes (*e.g., Myc, MDR, VEGF*) (10). Finally, *p53* mutant alleles that appear

normal for transactivation, growth suppression and apoptosis when ectopically expressed

at high levels in a tumor cell line, have been detected in breast and ovarian tumors

associated with *BRCA1* defects (11). Hence, detailed functional analysis of multiple

tumor *p53* alleles is expected to provide valuable information in predicting tumor state,

including aggressiveness and responsiveness to therapy.

We developed functional assays based in the yeast *Saccharomyces cerevisiae* that

provide for functional classification of p53 alleles using quantitative gene reporter assays

in a constant chromatin structure. The systems rely on *rheostatable* regulation of p53

expression and determine the transactivation capacity of p53 mutants relative to wild-

type p53 or the dominance potential of mutants when heterozygous with wild-type p53

using a large spectrum of p53 Response Elements (REs). The yeast functional assay

offers a practical means to develop a *p53 mutant functionality database* that collects the

functional fingerprints for all the p53 mutants relevant to cancer to be linked with the

IARC p53 mutation database. This information will become valuable in understanding

the correlation between p53 functional status and clinical outcome, thus providing

directions for effective patient management in the clinical setting.

RESULTS (BODY)

A detailed description of the experimental approach, results and the discussion of the implications of this work are presented in the appended publication (see Appendix 1). A general scheme of p53-regulated transcriptional targets and their functions is presented in Appendix 2, Figure 1. Figure 2 shows the distribution of p53 mutations reported in tumors along with the topological, functional and evolutionary conserved domains of the protein. Figures 3 A-C describe the yeast-based p53 functional assay we developed drawing in part from previously published works (12). Examples of the functional fingerprinting (both transactivation and dominance) of p53 mutant alleles reported in familial and sporadic breast cancers are presented in Appendix 3. A general strategy for the construction of any p53 mutant allele of choice based on our recently developed *delitto perfetto in vivo* mutagenesis system (13) is presented in Appendix 4, Figure 1A. A high-throughput experimental scheme for determining the transactivation capacity of any p53 mutants at many promoter elements is also presented in Figure 1B.

The following is a summary of the results described in the appended publication (Appendix 1).

1) Development of a panel of isogenic yeast strains containing p53 responsive promoters (pages 2-3 of Appendix 1).

26 different p53 REs were selected from p53-regulated promoters of genes involved in different biological pathways including cell cycle arrest, apoptosis (both mitochondrial and receptor pathway), DNA repair and regulation of p53 stability/activity. Simple REs that provides for a p53 tetramer binding site were integrated in the same chromatin locus to regulate the expression of the ADE2 (red/white) reporter gene.

2) Development of a tightly regulated p53 expression cassette in yeast based on a rheostatable *GAL1,10* promoter (page 4 and FIG.2 of Appendix 1).

The *GAL* promoter is repressed on glucose, while on raffinose a derepressed, higher basal transcriptional state is achieved (14). We observed that the p53 level on glucose is about 15 times lower than on raffinose based on densitometric analysis of Western blots. Varying the amounts of galactose inducer added to the raffinose medium lead to a gradual induction of the promoter over a wide range. The induction of p53 expression was approximately linear over the galactose range of concentration from 0% to 0.12%.

3) Rheostatable expression of wild-type *p53* revealed a broad range of transactivation capacities with the different REs (pages 4-6 and FIG. 3-4 of Appendix 1).

Transactivation of *ADE2* by p53 results in pink and white colonies, depending on the extent of induction of the *ADE2* reporter.

The relative ability of p53 to transactivate the reporter gene at various REs was examined by using different levels of galactose inducer and ranking the amount of galactose required for turning colonies from red to pink (= weak transactivation) and to white (= strong transactivation). The 26 *yAFM*-REs could be ranked for ability to be induced by different amounts of p53 using this phenotypic color assay. Surprisingly, there was as much as a 1000-fold difference in transactivation. Our results suggest that intrinsic DNA binding affinity, as well as p53 protein levels, are important contributors to p53-induced differential transactivation. We found that p53 had weak activity towards the apoptotic REs of the mitochodrial pathway of programmed cell death.

4) Transactivation capacity measured by color assay corresponds to *ADE2* mRNA levels (pages 6-7 and FIG. 6 of Appendix 1).

The red/pink/white phenotypic assay for assessing p53 gene-specific transactivation capacity and the impact of different levels of expression is simple and highly reproducible. However, the mechanism of red pigment accumulation and metabolism by the *ADE2* gene product to result in white colonies is unclear and might be affected by processes that are independent of p53.

We addressed the relationship between colony color and *ADE2* transcription using a quantitative PCR approach in real-time. These experiments revealed that there were comparable levels of *ADE2* expression from various REs in the absence of p53 protein and that the levels of mRNA induced by p53 at various REs reflected colony color, indicating a good correlation between the phenotypic assay and transactivation of the *ADE2* gene.

5) Statistical predictions of binding probability of the REs based on nucleotide usage do not correlate with the functional rank (pages 8 and 11 of Appendix 1).

Neither the number of non-consensus bases in the REs sequence nor statistical methods based on nucleotide usage [Heterology index (15) as well as MH-Algorithm (16)] predicted the wide variations in transactivation capacity we observed among the panel of p53 REs.

6) The CATG sequence at the center of a p53-dimer binding site greatly affects RE activity (pages 8 and 11 of Appendix 1).

We hypothesize that sequence-dependent structural features of the DNA greatly affect p53 activity in our *in vivo* system, as previously observed *in vitro* with purified p53 DNA binding domain and naked DNA RE (17). It is possible that upon interaction with DNA

RE sequences the p53-DNA complex undergoes conformational changes that significantly contribute to binding affinity and activity. Consistent with this we noticed that the four strongest REs have the 5'-CATG sequence at the junction between p53 monomer binding sites in both dimer sequences. Previous in vitro studies showed that the flexibility of the CATG sequence facilitates axial bending of the DNA upon interaction with p53 protein. The construction of artificial p53 REs with defined changes at the junction between monomer binding sites confirmed the important role of the CATG sequence in transactivation capacity.

7) Subtle changes in transactivation capacity are revealed in *p53* mutations associated with familial breast cancer (page 8 and Table 2 of Appendix 1).

The incidence of p53 mutations in familial breast cancer associated with germline BRCA1/2 mutations is nearly 70% as compared to around 30% for sporadic breast cancer. Moreover, the spectrum of mutations appears to be different(18). Interestingly, a subset of BRCA1-associated p53 mutant alleles appeared wild type in mammalian functional assays (11) and 4 of the mutants, T150I, G199R, R202S, and S215C, were also wild type in a yeast transactivation assay at high p53 expression (9). We determined the transactivation capacity of these four alleles relative to wt p53 using the rheostatable *GAL* promoter system for p53 expression. Only under conditions of low expression we were able to detect subtle changes in transactivation comprising both enhanced and reduced activity for several REs

8) Functional fingerprinting of p53 mutant alleles reported in sporadic and familial

breast cancer (Figure 1 and 2 of Appendix 3).

A larger panel of p53 mutant alleles and REs was analyzed with the yeast system in order

to develop functional fingerprints. Both transactivation capacity and dominance potential

were determined.

Forty p53 mutations were tested including DNA contact mutants, mutation hotspots in

breast and other cancer types, mutations preferentially associated with familial breast

cancer, and novel alleles in the L1 loop of the DNA binding domain. Figure 1 in

Appendix 3 contains a graphical representation of the transactivation capacity of these

alleles relative to wt p53. The yeast functional assay at variable p53 expression and with

many p53 response elements revealed both subtle and dramatic changes in transactivation

including increased activity compared to wt p53. A significant fraction of p53 mutants

retain partial function in this analysis, including some tumor hotspots. Interestingly, the

group of p53 alleles preferentially associated with familial breast cancer showed a unique

functional fingerprint characterized by subtle increase and decrease in activity with

several REs.

The analysis of the dominance potential (Figure 2 in Appendix 3) of p53 mutants when

expressed at equal low/variable levels with wt p53 was sensitive to the p53 gene dosage

since it distinguished the presence of one versus two wt p53 alleles. Different degrees of

dominance were observed with the tumor alleles. Consistent with the transactivation

results the p53 mutants associated with breast cancer were not dominant although some

caused a reduction of gene dosage phenotype.

KEY RESEARCH ACCOMPLISHMENTS

1) **Development of a panel of isogenic yeast strains containing p53 responsive promoters and of a rheostatable *GAL1,10* promoter system providing for tight regulation of p53 expression.**

2) **Determination of the intrinsic transactivation capacity of wild type p53 towards its many response elements (REs) using an *in vivo* system with constant chromatin structure.** A broad range of transactivation capacity was detected with low activity particularly for apoptotic REs of the mitochondrial pathway.

3) **Poor correlation between *in vivo* transactivation capacity and predicted binding probabilities based on statistical analyses.** Available algorithms estimating binding probabilities of p53 to its many REs did not predict the result of the functional assay. The difference in intrinsic transactivation capacity are likely to be influenced by mutually induced conformational changes of the DNA target site and the p53 protein upon recognition and interaction with a RE.

4) **Identification of subtle changes in transactivation capacity comprising both reduced and enhanced activities in a group of rare *p53* mutations associated with familial breast cancer, previously classified as wild type.**

5) **Use of the yeast-based assays for functional fingerprinting of p53 mutant alleles.** A significant fraction of p53 mutant alleles associated with cancer retain partial transactivation. Loss-of-function alleles exhibited different degrees of dominance over wt p53 when heterozygous.

6) **Development of the concept of** *the p53 mutants functionality database.* The p53 functional assays developed in yeast provide means to develop a *functionality database* of all tumor p53 mutations associated with breast and other cancers with implications to ascertain the correlation between p53 functional status and tumor aggressiveness and responsiveness to therapy.

REPORTABLE OUTCOMES

The results of our work supported by the DOD Concept Award have been presented at international meetings and in the publications listed below:

<u>presentation at meetings</u>

1) A sensitive *in vivo* system determines the transactivation specificity and selectivity of human p53 alleles at many promoter elements. -Inga, Storici, Bouma, Darden, and Resnick, poster presentation-

93rd Annual Meeting, American Association for Cancer Research, April 6-10, 2002, San Francisco, CA, USA

2) Functional fingerprints of human p53 mutants using quantitative yeast-based transactivation assays reveal specific changes in p53 activity that may be relevant to tumor biology. -Inga, Storici, Darden, and Resnick, oral presentation-

2002 Yeast Genetics and Molecular Biology Meeting, July 30-August 4, 2002, Madison, WI, USA

3) Functional fingerprints of human p53 mutants using quantitative yeast-based

transactivation assays reveal specific changes in p53 activity that are relevant to tumor

biology. -Inga, Storici, Bouma, Liu, King, Monti, Darden, Fronza, and Resnick, poster

presentation-

Era of Hope Department of Defense Breast Cancer Research Program Meeting,

September 25-28, 2002, Orlando, FL, USA

publications

1) A. Inga, F. Storici, T. A. Darden and M. A. Resnick (2002). Differential

transactivation by the p53 transcription factor is highly dependent on p53 level and

promoter target sequence. MCB, in press -see Appendix 1.

2) A. Inga, P. Monti, B. Bouma, G. Fronza, and M.A. Resnick. Functional fingerprinting

of tumor p53 alleles using quantitative yeast-based assays. -in preparation-

CONCLUSIONS AND FUTURE DIRECTIONS.

We developed new tools providing for the functional profiling of p53 alleles reported in

breast and other cancers both in terms of transactivation capacity and dominance over wt

p53. The results presented in Appendix 3 together with other unpublished observations

(Storici *et al.*, manuscript in preparation) strongly suggest that many p53 mutations

associated with cancer are likely to retain partial transactivation function *in vivo*.

Although several classification methods for p53 mutants have been attempted our

findings indicate that it is not presently possible to predict *a priori* the behaviour of a

mutant p53 protein. The yeast functional assays provide an important contribution to the

functional classification of p53 alleles. It appears that the combination of low and

variable expression of p53 alleles and the assessment of the transactivation capacity using many p53 response elements provides a greater sensitivity for the functional classification of p53 mutants than the standard yeast assays using high constitutive p53 expression. When combined with the efficient method for constructing p53 alleles using *in vivo* mutagenesis (13) (see Appendix 4, Figure 1A) and with the rapid screening system depicted in Figure 1B of Appendix 4, these tools allow for rapid development of p53 functional fingerprints.

Hence, the yeast functional assay offers a practical means to develop a *p53 mutant functionality database* that enables the functional fingerprints for all the p53 mutants relevant to cancer to be linked with the IARC p53 mutation database. Particularly in sporadic breast cancer, published and ongoing studies focus on the correlation between p53 status and clinical outcome. However, the functionality of the tumor p53 alleles has never been carefully considered in these analyses which yielded contrasting results. Recent reports highlighting the complex functional interactions between Estrogen Receptor, BRCA1 and p53 (19) further strengthen the value of detailed functional analyses of p53 mutant alleles reported in breast cancer. We propose that a *p53 mutant functionality database* will become valuable in understanding the correlation between p53 functional status and tumor aggressiveness and responsiveness to therapy, and may provide directions for effective patient management in the clinical setting.

The financial support from the Department of Defense Breast Cancer Research Program has been instrumental in the development of these yeast-based functional-assay systems. We hope that future support would allow us to focus our investigation on elucidating the prognostic value of p53 functional status in breast cancer.

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Vol. 22, No. 24   DOD Research Report Appendix 1

Differential Transactivation by the p53 Transcription Factor Is Highly Dependent on p53 Level and Promoter Target Sequence

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Q: A

Little is known about the mechanisms that regulate differential transactivation by p53. We developed a system in the yeast *Saccharomyces cerevisiae* that addresses p53 transactivation capacity from 26 different p53 response elements (REs) under conditions where all other factors, such as chromatin, are kept constant. The system relies on rheostatable regulation of p53 expression. The p53 transactivation capacity toward each 20- to 22-bp-long RE could be ranked by using a simple phenotypic assay. Surprisingly, there was as much as a 1,000-fold difference in transactivation. There was no correlation between the functional rank and statistical predictions of binding energy of the REs. Instead we found that the central sequence element in an RE greatly affects p53 transactivation capacity, possibly because of DNA structural properties. Our results suggest that intrinsic DNA binding affinity and p53 protein levels are important contributors to p53-induced differential transactivation. These results are also relevant to understanding the regulation by other families of transcription factors that recognize several sequence-related response elements and/or have tightly regulated expression. We found that p53 had weak activity towards half the apoptotic REs. In addition, p53 alleles associated with familial breast cancer, previously classified as wild type, showed subtle differences in transactivation capacity towards several REs.

n*   Complex modulation of gene expression is an integral component of specific cellular responses to environmental stresses. This is generally achieved through the activation and regulation of transcription factors by the integrated action of multiple signaling pathways (78). The p53 protein is a remarkable example of a biochemical signal integrator whose transactivation activity is critical in the prevention of cellular transformation (46). Its function may be altered in nearly all human cancers, and an average of 50% of tumors express mutated forms of the protein with single amino acid changes that in some cases retain native conformation and partial function (10, 48).

Wild-type p53 can modulate the transcription of a vast number of target genes that play a role in diverse processes, including cell cycle control, apoptosis, senescence, differentiation, and DNA repair as well as its own turnover and activity (77). Differential transactivation by p53, in terms of both selectivity and kinetics of gene activation and repression, is important in determining which of these various processes is elicited, particularly with respect to the choice between cell cycle arrest and apoptosis (41, 69). However, little is known about the mechanisms that determine the in vivo specificity of p53 responses (44, 78).

Specific DNA binding by wild-type p53 is essential to its role as a tumor suppressor. This view is supported by the presence of p53 response elements (REs) in p53-regulated genes, the strong selection for inactivation of transactivation function during tumorigenesis, and the spectrum of p53 mutations observed in cancer cells (43, 79). The consensus sequence of a

p53 RE consists of two copies of a degenerate 10-bp motif, 5′-RRRCWWGYYY-3′, with a spacer of up to 13 bp (21) that provide for the binding of a p53 tetramer (50). Many potential p53 targets are being identified through genome-wide sequence analysis and expression profiling in response to expressed p53 (40, 80, 85, 88).

There are many factors that might contribute to differential transactivation of various genes by p53. These include the individual sequence differences between REs, number of REs, and location of an RE with respect to the transcription start site (20, 62). The amount of nuclear p53 protein and the regulation of its DNA binding capacity may also be factors in differential transactivation (15, 63, 88). In addition to p53 levels (1, 58), posttranslational modifications may directly influence p53 DNA binding specificity, providing a link between a particular stress signal, an upstream p53 modifier, and an induced response (2, 13, 19). Cell type and physiology could also modulate p53 activity through gene-specific or general coactivator proteins (27, 65).

The roles of the structural organization of the promoters containing p53 REs and locus-specific features of chromatin assembly and remodeling in regulating p53 transactivation have recently been investigated. p53 is latent (needs posttranslational modifications) for binding with an RE in naked DNA but is active for in vitro binding to the same RE when chromatin is present (23). Based on in vivo chromatin immunoprecipitation, p53 is an active DNA binding protein (39, 69). In addition, acetylation of chromatin is important for p53 transactivation (3), while acetylation of p53 does not influence its DNA binding activity. The p53 protein may bind to a RE and recruit coactivator or corepressor proteins whose chromatin-remodeling activities (e.g., histone acetylation and deacetylation) modulate transcription (23, 49, 81). Although the actual

UAS +pADE2 → ade2,1 ORF

Chr XV

↓ Two steps PCR-based
↓   gene replacement

pCYC1   ADE2 ORF

CYC1 promoter w/o UAS

delitto perfetto in vivo mutagenesis
Step 1 = CORE integration

CORE

pCYC1   ADE2 ORF

CORE   pCYC1   ADE2 ORF

p53 RE

Step 2 = CORE replacement by
oligonucleotides

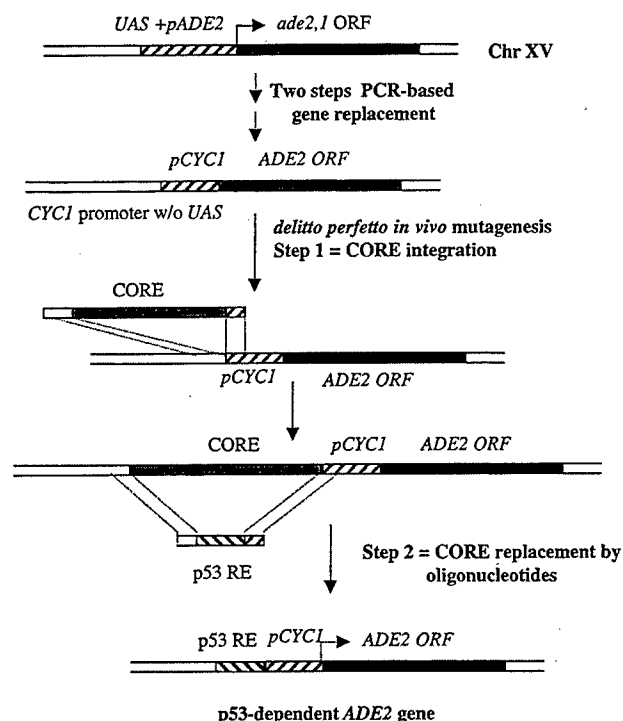p53 RE *pCYC1* → ADE2 ORF

p53-dependent *ADE2* gene

FIG. 1. Construction of the p53-dependent *ADE2* locus in the yAFM yeast strain. The *ade2,1* open reading frame (ORF) and promoter on chromosome (Chr) XV of the yIG397 strain was first replaced with a wild-type *ADE2* under control of a minimal *cyc1* promoter lacking upstream activating sequences. The delitto perfetto system of in vivo mutagenesis (see text for details) was then employed to rapidly generate a panel of isogenic yeast strains whose *ADE2* gene is under p53 transcriptional regulation via different p53 REs.

impact on gene expression is likely to be influenced by target locus-specific features, p53 protein availability and intrinsic relative affinity of binding to p53 REs can be anticipated to play an important role in achieving differential transactivation.

In this work we address the role that the RE sequence and protein level can have in the differential transactivation by p53 at different REs. To accomplish this, we developed an in vivo system based in the model eukaryote, the yeast *Saccharomyces cerevisiae*, that relies on (i) a wide range of p53 protein expression levels from a tightly regulated rheostatable promoter, (ii) the use of a p53-dependent reporter gene located in single copy at its native chromosomal locus, and (iii) comparison of the transactivation activities of 26 responsive promoters, each comprising a single p53 tetramer binding site RE in an otherwise isogenic context. The functional analysis system has been extended to four p53 alleles associated with familial breast cancer that appeared to be indistinguishable from wild-type p53 in previous mammalian and yeast assays (11, 67).

MATERIALS AND METHODS

Construction of isogenic yeast strains containing p53-responsive promoters. The *S. cerevisiae* yAFM-RE strains were constructed from strain yIG397 (*MATa ade2-1 leu2-3,112 trp1-1 his3-11,15 can1-100 URA3::3xRGC::p-cyc1::ADE2:: ura3-1*) (24) as illustrated in Fig. 1. First we removed the p53 reporter construct integrated at the *URA3* locus by selecting Ura⁻ cells on 5-fluoro-orotic acid plates (Toronto Research Chemicals Inc., Toronto, Ontario, Canada). The loss of the *ADE2* cassette was confirmed by colony PCR. Genomic DNA from strain

1
Q: B

yIG397 was used as a template to amplify the *cyc1::ADE2* promoter-reporter construct by using a pair of primers with tails containing restriction sites for cloning. The *cyc1* promoter fragment corresponds to chromosome X positions 525782 to 526025. The resulting 2-kb fragment was then cloned into plasmid pUC18 to generate pUC-*cyc1ADE2*. This was used as a template to generate a PCR fragment targeting the yeast *ADE2* locus and containing the *cyc1* minimal promoter and the wild-type *ADE2* open reading frame, but no p53 RE. This fragment was integrated by exploiting homologous recombination at chromosome XV between positions 564474 and 566541. Besides the *ade2-1* open reading frame, 350 bp of the *ADE2* promoter upstream of the transcription start site (position 566190) was replaced by the integration. This strain, named yAFM, was red on plates with low adenine levels, indicating that upstream activating sequences were required for effective *ADE2* transcription. Next we used a recently developed in vivo site-specific mutagenesis system known as delitto perfetto (68) to create p53-responsive *cyc1* promoters. The CORE cassette (CO, counterselectable, *KLURA3*; RE, reporter, *KanMX4*, G418 resistance gene) was amplified and integrated immediately upstream from the minimal *cyc1* promoter. Finally, the CORE cassette in the strain now named yAFM-CORE was replaced by using a pair of oligonucleotides containing the 20-bp p53 RE flanked by 40-bp homology tails. In order to insert p53 REs longer than 20 bases (i.e., with spacers or more than one tetramer binding site), we used a pair of oligonucleotides with a 30-base overlap at their 3' sites that were extended in vitro before transformation in yeast as described previously (68). CORE replacements were confirmed by colony PCR and automated DNA sequencing (Applied Biosystems, Foster City, Calif.). Each isogenic yAFM-RE strain is identified by the name of the specific p53 RE (Table 1). All yAFM-RE strains without p53 expression **T1** vector were red on plates with low adenine levels. The sequences of all primers and details on strain construction are available upon request.

Phenotypic transactivation assay with variable p53 expression. The transactivation of the *ADE2* gene, which is dependent on p53 activity, results in the color of colonies changing from red to pink and to white (24). Plasmid pLS89 (66), coding for the human wild-type p53 cDNA under the inducible *GAL1-10* promoter, was introduced into the yeast strains by lithium acetate transformation (29). Transformants were purified onto selective glucose plates (lacking tryptophan) containing a high adenine level to overcome the growth defect caused by incomplete adenine biosynthesis. The transactivation assay was then performed on plates with a low adenine level (5 mg/level) to allow color detection, using three independent transformants of each yAFM-RE strain. In order to modulate the expression of p53 protein from the *GAL* promoter, we used a combination of different sugars in the plate assay. Two percent raffinose (Sigma, St. Louis, Mo.) was used as a basic carbon source to keep the promoter in a derepressed state; variable amounts of galactose (Pfanstiehl, ●●●, Ill.) were added to induce dif- **AQ: C** ferent levels of transcription. A glucose plate was also used to achieve minimal p53 expression (the *GAL* promoter is actively repressed on glucose). Thus, each transformant was streaked to 12 different low-adenine plates allowing different amounts of transactivation (see Fig. 3). The plates were incubated at 30°C for 3 days before the colony color was scored.

Western blotting. To verify that variable amounts of galactose led to a fine-tuning of p53 protein expression, yAFM transformants were grown overnight in selective glucose medium. Cells were washed and diluted to 10⁶/ml in raffinose-galactose medium, using the same concentrations as in the plate assay (see Fig. 2 for concentrations). A high concentration of adenine (200 mg/liter) was used to allow for equal growth of all strains irrespective of the extent of p53-induced *ADE2* transactivation. Proteins were extracted measured and blotted as previously described (37). p53 was detected by using pAb1801 and DO-1 monoclonal antibodies (Santa Cruz, Santa Cruz, Calif.). Relative p53 protein induction was determined by film scan and densitometric quantification with the free National Institutes of Health image software Scion Image 1.62. **AQ: D**

ADE2 **mRNA expression measurements by real-time quantitative RT-PCR.** To quantitate p53-dependent transactivation, the *ADE2* mRNA was measured from aliquots of the same cultures used to prepare the protein extracts. The ratio between *ADE2* and *ACT1* (actin) mRNAs was determined by using quantitative reverse transcription-PCR (RT-PCR) approaches. Total RNA from 4 ml of overnight cultures was prepared by using the RNAeasy kit and protocol (Qiagen, Valencia, Calif.). The RNA preparations were treated with DNase I to eliminate any possible DNA contamination. Total cDNA was prepared from 200 ng (final concentration, 20 ng/µl) of total RNA using random hexamers (Applied Biosystems, Foster City, Calif.) and MultiScribe reverse transcriptase (Applied Biosystems). Different amounts of cDNA (2 µl of 1:100, 1:10, and 1:1 dilutions) were used for the specific amplification of *ADE2* and *ACT1*. The primer pairs were chosen by using the ABI PRISM 7700 sequence detection system software (Applied Biosystems). Quantification of the *ADE2/ACT1* ratio was obtained by using both the SYBR Green kit (Applied Biosystems) and TAQMan chemistry

TABLE 1. DNA sequence, ranking based on transactivation activity, deviation from consensus, and predicted affinity of 22 p53 REs from p53-regulated genes and 4 artificial REs

Tetramer (dimer of dimer) p53 binding sequence[a] — arrows indicate monomer binding sites and orientation: → ← → ←

Sequence type	R(1)	R(2)	R(3)	C(4)	W(5)	W(5)	G(4)	Y(3)	Y(2)	Y(1)	N	R(1)	R(2)	R(3)	C(4)	W(5)	W(5)	G(4)	Y(3)	Y(2)	Y(1)	Name and function[b] of gene (reference)	No. of non-consensus bases	HI[c] T	HI[c] B
Consensus	R	R	R	C	W	W	G	Y	Y	Y	N	R	R	R	C	W	W	G	Y	Y	Y				
Mammalian gene	C	A	A	C	A	T	G	T	T	G		G	G	A	C	A	T	G	T	T	C	P21-5′,C (23)	2	13.6	14.6
	T	G	A	C	A	T	G	C	C	C		A	G	G	C	A	T	G	T	C	T	P53R2,D (70)	1	10.2	8.1
	G	G	G	C	A	T	G	T	A	C		A	A	A	C	A	T	G	T	C	A	m-FAS,A (53)	2	10.8	15.7
	G	A	A	C	A	T	G	T	C	T		A	A	G	C	A	T	G	C	T	G	GADD45,D (42)	1	12.8	13.9
	T	G	G	C	T	T	G	T	C	A		G	G	G	C	T	T	G	T	C	C	HFAS,A (53)	2	10.4	11.5
	T	A	G	C	AT	TA	G	C	C	C		A	G	A	C	A	T	G	T	C	C	14-3-3 σ,C (32)	1[d]	10.4	9.7
	G	A	A	C	A	A	G	T	C	C		G	G	G	C	A	T	A	T	G	T	PCNA,D (83)	2	14.2	21.9
	T	C	T	C	T	T	G	C	C	C		G	G	G	C	T	T	G	T	C	G	AIP1,A (57)	4	18.8	20.5
	C	T	G	C	A	A	G	T	C	C		T	G	A	C	T	T	G	T	C	C	PUMA,A (84)	3	14.5	14.5
	A	G	G	C	T	T	G	C	C	C		G	G	G	C	A	G	G	T	C	T	Cyclin G,C (87)	1	10.6	12.3
	A	G	G	C	T	T	G	C	C	C		C	G	G	C	A	A	G	T	T	G	NOXA,A (56)	2	14.3	14.9
	G	A	A	G	A	A	G	A	C	T		G	G	G	C	A	T	G	T	C	T	P21-3′,C (23)	2	15.9	21.2
	G	G	T	C	A	A	G	T	T	G		G	G	A	C	A	C	G	T	C	C	MDM2-RE1,O (86)	3	17.5	23.5
	G	G	A	C	A	A	G	T	C	T		C	A	A	C	A	A	G	T	T	C	PA26,C (75)	1	13.2	14.4
	A	G	A	C	A	A	G	C	C	T		G	G	G	C	G	T	G	G	G	C	BAX-B,A (71)	3	15.6	21.6
	G	A	G	C	T	A	A	G	T	C	C	T	G	A	C	A	T	G	T	C	T	MDM2-RE2,O (86)	3	20.0	22.6
	G	G	A	C	T	T	G	T	C	T		G	A	G	C	G	C	G	T	G	C	cFOS,O (22)	3	18.1	22.4
	A	C	A	C	T	T	G	T	C	A		T	A	G	C	A	A	G	A	C	T		4	23.7	25.8
	G	G	A	C	T	T	G	C	C	T		G	G	C	C	T	T	G	C	C	T	RGC,O (66)	1	14.7	12.1
	A	A	A	C	A	A	G	C	C	A	C	C	A	A	C	A	T	G	C	T	T	IGF-BP3 A,A (7)	2	16.0	14.5
	C	A	G	C	T	T	G	C	C	C		A	C	C	C	A	T	G	C	T	C	PIG3,A (76)	3	18.9	17.7
	T	C	A	C	A	A	G	T	T	A	G	A	G	A	C	A	A	G	C	C	T	BAX-A,A (71)	3	18.3	16.6
	G	G	G	C	A	A	G	A	C	C		T	G	C	C	A	A	G	C	C	T	IGF-BP3 B,A (7)	3	15.4	19.6
Artificial	G	G	G	C	A	T	G	T	C	C		G	G	G	C	A	T	G	T	C	C	Con-A,O (52)	0	6.2	9.3
	G	G	G	C	T	A	G	T	C	C		G	G	G	C	T	A	G	T	C	C	Con-B,O	0	11.5	13.9
	G	G	G	C	A	A	G	T	C	T		G	G	G	C	A	A	G	T	C	T	Con-C,O (16)	0	9.4	12.0
	G	G	A	C	A	T	G	C	C	T		G	G	C	C	A	T	G	C	C	T	Mut-RGC,O	1	11.7	11.0

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[a] The arrows indicate the monomer binding sites and their orientation, underlining indicates nucleotides that deviates from the consensus, and boldface indicates ●●●●●.   Q: I

[b] C, Cell cycle control; D, DNA repair; A, apoptosis; O, other.

[c] Based on a weighted consensus derived from published work by Tokino et al. (73) (transactivation assay [T]) and el-Deiry et al. (21) (in vitro binding assay [B]) and the equation developed by Berg and von Hippel (4). See text.

[d] Two additional weak bases separate the first two monomer binding sequences.

(Applied Biosystems). Both protocols gave overlapping results. The first method quantifies the amount of amplification but cannot discriminate between specific and nonspecific PCR products. The second procedure exploits two fluorescent probes that are specific for the templates under examination, allowing for multiplex PCR. The amount of amplicons produced is measured in real time (cycle by cycle) in the ABI 7700 thermal cycler (Applied Biosystems), so that the linear fraction of the amplification reaction is used for quantification. The $ADE2/ACT1$ mRNA ratio was obtained by the comparative C_T method by using the C_T values for the VIC and FAM probes calculated from the linear fraction of the amplification reaction, corrected by the spectral compensation for multiplex PCR (Applied Biosystems). The ΔC_T values for the three different cDNA dilutions used were independently computed and averaged. The standard deviations presented in Fig. 4 represent the variation observed between three independent RNA-cDNA preparations examined by QT-PCR. The sequences of all primers and probes and details of the amplification reactions are available upon request.

Q: E

Q: F

RESULTS

Development of a panel of isogenic strains containing p53-responsive promoters. We sought to develop an in vivo system to systematically evaluate the contributions of RE sequence and p53 protein level to differential transactivation. This required the ability to assess the capacities for transactivation by various amounts of p53 protein towards many REs inserted at the same position within a reporter gene. Yeast-based p53

functional assays have provided valuable tools for screening tumor samples, classifying mutant p53 alleles, and structure-function studies (8, 9, 14, 38). High-level, constitutive expression of wild-type p53, but not tumor mutants, could activate a promoter containing a p53 RE in yeast (66). Using an inducible p53 expression system, we had previously identified various functional classes of p53 mutant alleles (36). Moreover, at low expression levels, wild-type p53 was more active with RGC and P21 REs than with a BAX RE (37). However, because of differences in strain backgrounds and structural arrangements of the REs, these comparisons were qualitative and an evaluation of differential transactivation could not be addressed.

To explore differential transactivation by p53, we developed a new set of isogenic haploid yeast strains based upon the *ADE2* red-white p53 reporter system initially described by Flaman et al. (24). Presented in Fig. 1 is a general system for incorporating REs into a promoter. We utilized our recently developed two-step in vivo mutagenesis system, termed delitto perfetto (68), to rapidly generate a panel of yeast strains with modified p53-responsive *CYC1* promoters. Briefly, the wild-type *ADE2* open reading frame was integrated into the chromosome, replacing the *ade2,1* allele in the yIG397 background
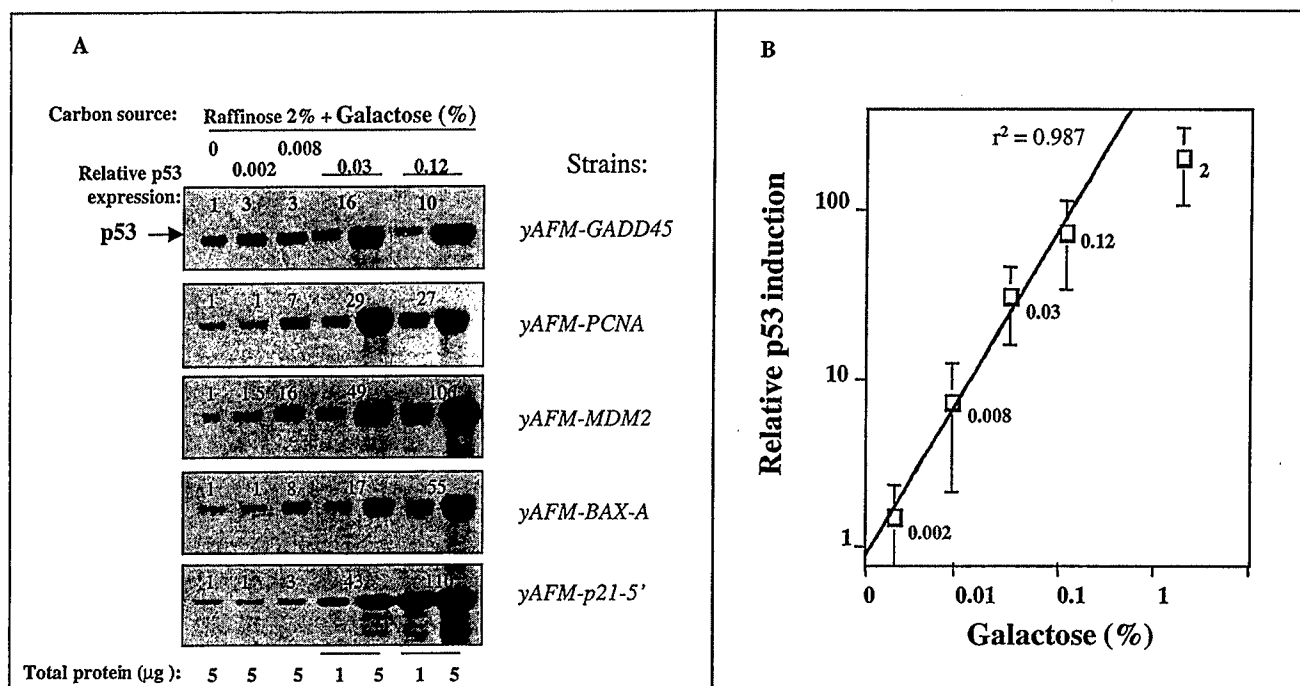
FIG. 2. Regulatable expression of p53 protein by using a rheostatable *GAL1* promoter. (A) pLS89 (*GAL1*::wild-type p53) transformants of the indicated yAFM strains were grown overnight in selective glucose medium, washed, diluted, and grown for 24 h in selective medium containing excess adenine and either raffinose or raffinose plus increasing amounts of galactose (indicated above the lanes). Different amount of protein extracts were loaded (indicated below the lanes). p53 was detected by Western blotting (with pAb1801 and DO-1). p53 induction relative to the level detected with raffinose was determined by densitometric analysis, taking into account the different amounts of protein loaded and averaging the results of the two different measurements in the case of the 0.03 and 0.12% galactose cultures. The p53 induction relative to raffinose cultures is shown for each strain. The variation in the amount of p53 among the various strains after growth on raffinose was equal to or less than threefold. (B) Relative p53 expression as a function of galactose concentration for five isogenic yAFM-RE strains. The standard deviations, the linear curve fit up to 0.12% galactose, and the correlation coefficient are shown.

(see Materials and Methods). The *ADE2* promoter was also replaced with a minimal *CYC1* promoter that lacked upstream activating sequences. Hence, *ADE2* transcription is inactive, and the strain, named yAFM, is red in medium containing low levels of adenine due to the interruption of adenine biosynthesis at the step prior to the one catalyzed by Ade2. Applying the delitto perfetto approach, a CORE cassette (CO, counterselectable marker; RE, reporter gene) was inserted by homologous recombination upstream of the minimal *CYC1* promoter, and then the cassette was replaced by using double-stranded oligonucleotides containing a specific p53 RE. Using this approach, we created 22 p53 REs from mammalian p53-regulated genes plus four artificial sequences (Table 1). The correct RE insertions were confirmed by DNA sequencing. At least two independent isolates for each RE, named yAFM-RE, were tested in the functional assay. With the exception of the c-*fos* RE (i.e., yAFM-cFOS), all of the RE sequences cloned upstream of the *CYC1* promoter contained a single 20- to 22-bp p53 tetramer binding site. Most of the REs have no spacer between the dimer binding sites. Table 1 describes the actual sequence of each element along with the mammalian gene name from which it was derived.

Rheostatable expression of p53 protein controlled by the *GAL1-10* promoter. A single-copy centromere plasmid, pLS89, containing the human wild-type p53 cDNA under control of the *GAL1-10* promoter was transformed into the yAFM-RE

strains. The *GAL* promoter is repressed on glucose, while on raffinose a derepressed, higher basal transcriptional state is achieved (34). The p53 level on glucose is about 15 times lower than that on raffinose (data not shown). When galactose is added to the raffinose medium, the promoter can be induced to a high level. In order to establish that the system was rheostatable for p53 protein expression, we determined p53 levels after growth in medium containing raffinose plus low levels of galactose (Fig. 2). The amount of p53 was quantified by using densitometric scans of Western blots (Fig. 2A). The levels of p53 protein were examined in five isogenic yAFM-RE strains, providing a mean relative p53 expression as a function of galactose concentration (Fig. 2B). The induction of p53 expression was approximately linear over the range of galactose concentration from 0 to 0.12%. The p53 level after induction with 2% galactose (see also Fig. 5B) increased only two to three times relative to that with 0.12% galactose.

Rheostatable expression of p53 reveals a broad range of transactivation capacities. The relative abilities of p53 to transactivate the reporter gene at various REs were examined by using different levels of galactose inducer and ranking the amount of galactose inducer required for colonies to become pink or white. The various yAFM-RE transformants containing the p53 expression vector were streaked out for single colonies onto a series of 12 plates (which select for the vector) containing either glucose, raffinose, or raffinose plus different

F2

glucose   **raffinose**   **0.001% galactose**
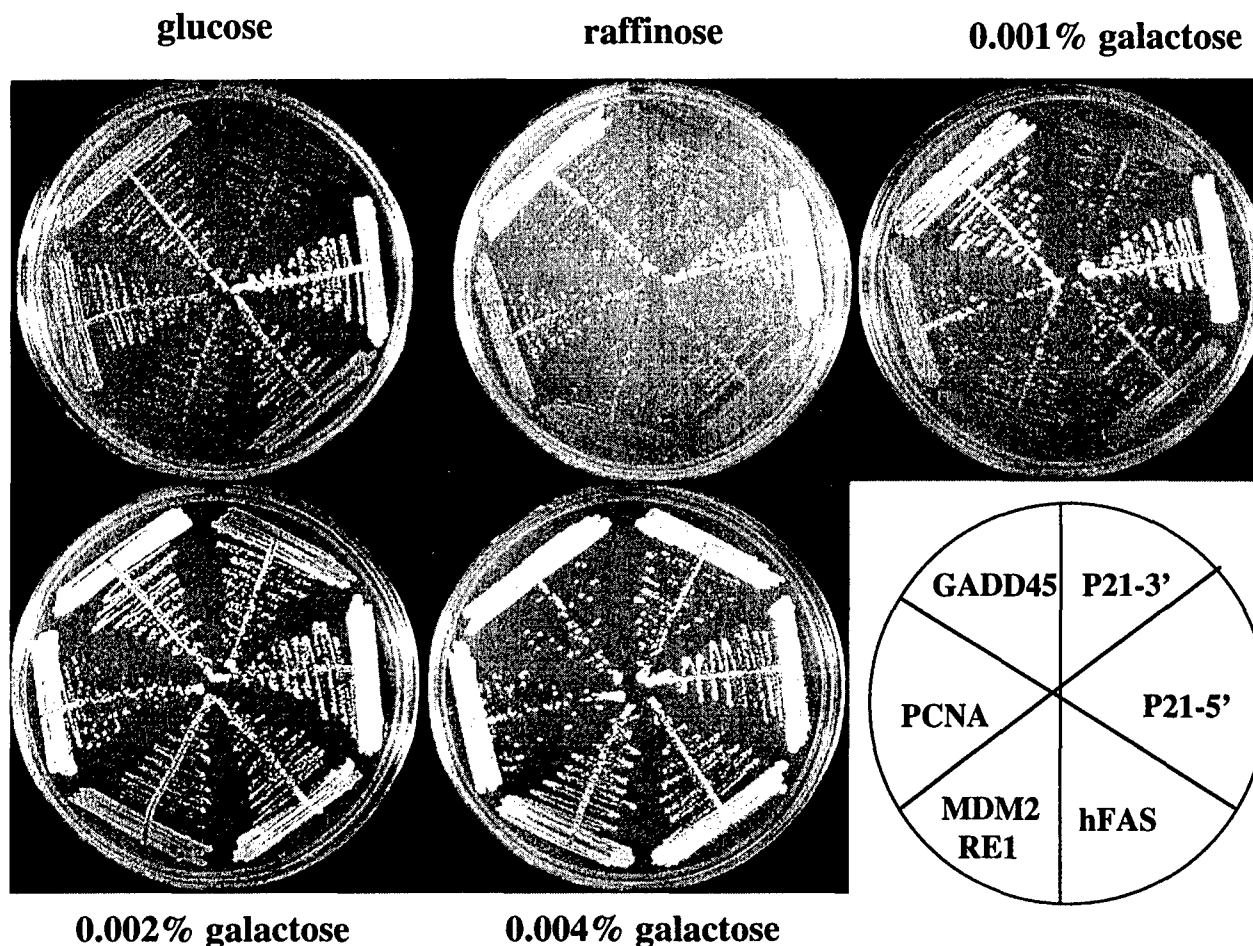

0.002% galactose   **0.004% galactose**

FIG. 3. Variable expression of human wild-type p53 and transactivation capacities at different p53 REs. Purified isogenic yAFM transformants with the *GAL1*::wild-type p53 expression vector were streaked out on plates containing raffinose as a carbon source plus increasing amount of galactose to achieve variable p53 expression. A low level of adenine in the medium was used in order to assess p53-dependent transactivation of the *ADE2* gene. Colonies turned from red to pink and to white at different amounts of galactose, indicating variable activity towards the different p53 REs.

amounts of galactose (increasing by factors of 2, i.e., 0.001, 0.002, 0.004, 0.008, 0.016, 0.032, 0.12, 0.25, 0.5, and 2%). A small amount of adenine (5 mg/liter) was included to enable growth of the *ade2* mutant cells to small red colonies if there was insufficient transactivation. Transactivation of *ADE2* by p53 results in pink and white colonies, depending on the extent of induction. If the transactivation capacity with all p53 REs is the same, colony colors would be expected to change from red to pink to white at comparable levels of galactose. Instead, the responses often differed between REs based on level of galactose required to produce pink or white colonies. For example, the yAFM-P21-5′ strain was nearly white on glucose plates, as seen in Fig. 3, indicating high activity by basal levels of p53 toward this element. Strains such as those containing m-cFOS or IGF-BP3 B remained pink or red at maximum p53 expression (2% galactose), as shown in Fig. 4.

The 26 yAFM-REs could be ranked for ability to be induced by different amounts of p53 by using this phenotypic color assay. Since the relative p53 expression was measured under the same culture conditions and appeared to be linear over a broad range ($r^2 = 0.987$) (Fig. 2B), the relative amount of p53

protein required for a phenotypic change (pink or white) could be determined. This allowed us to rank p53 transactivation capacity towards the 26 REs as the minimum level of p53 required to produce changes in colony color. (As described below, color correlates well with level of *ADE2* transcription.) Furthermore, since all strains were isogenic, the transactivation capacities directly reflect the combination of the DNA binding affinity and the ability to transactivate from individual REs by p53.

The functional ranking of p53 transactivation at the various REs of p53-regulated genes is presented in Fig. 4; P21-5′ is the strongest, and the IGF-BP3 box B is the weakest (the Con and Mut-RGC response elements are described below). In addition to P21-5′, five yAFM-REs (p53R2 to hFAS) showed some transactivation at the basal levels of expression on glucose-only plates. All other strains were red on both glucose and raffinose. For 15 of the yAFM-REs (p53R2 to MDM2/RE1), white colonies could be detected at some level of p53 induction. For 12 of the latter (p53R2 to cyclin G), colonies appeared white (or nearly white as in the case of hFAS, PUMA, PCNA, and cyclin G) when there was less than a 20-fold induction of p53 protein
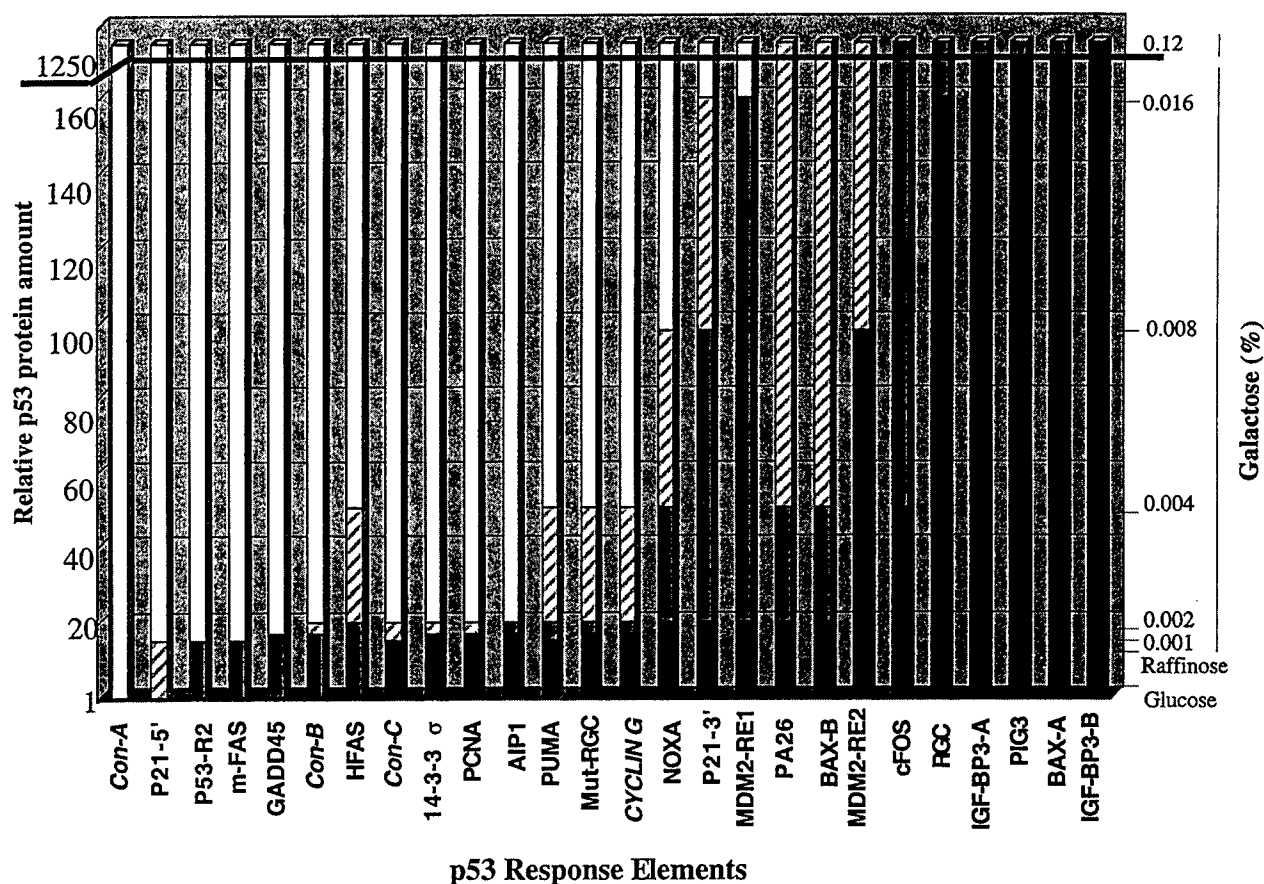
FIG. 4. Functional ranks of 26 p53 REs. The results of the phenotypic transactivation assay at variable p53 protein expression levels are summarized. The pattern of change in colony color with increasing p53 expression is shown for every RE. Black bars, red colonies; gray bars, pink colonies; hatched bars, light pink colonies; white bars, white colonies. The y axis on the left indicates the p53 protein induction relative to the level measured on glucose. The y axis on the right indicates the galactose concentrations used in the plate assay.

relative to the level on glucose. The yAFM-NOXA, -P21-3′ and -MDM2/RE1 elements enabled some p53 transactivation with, a 20-fold relative increase in induction of p53, based on the appearance of pink colonies; much more induction was required to produce white colonies. The remaining nine elements (PA26 to IGF-BP3 box B) did not enable high levels of transactivation, since the colonies did not turn white even under conditions of maximum p53 expression. Except for the IGF-BP3 box B RE, which was completely inactive, the colonies exhibited a light to dark pink color on 2% galactose.

Transactivation capacity measured by color assay corresponds to *ADE2* mRNA levels. The red-pink-white phenotypic assay for assessing p53 gene-specific transactivation capacity and the impact of different levels of expression is simple and highly reproducible. However, the mechanism of red pigment accumulation and metabolism by the *ADE2* gene product to result in white colonies is unclear and might be affected by processes that are independent of p53. We therefore addressed the relationship between colony color and *ADE2* transcription. The *ADE2* mRNA levels were measured relative to actin mRNA (*ACT1*) by using a real-time RT-PCR approach (see Materials and Methods). First, we checked that there were comparable levels of *ADE2* expression from various REs in the absence of p53 protein. Two of the strongest (yAFM-P21-5′

and -p53R2) and the two weakest (BAX-A and IGF-BP3 box B) p53-responsive strains were compared. The red yIG397 strain with a wild-type *ADE2* promoter in front of the *ade2,1* allele was also included (Fig. 5A). Total RNA was prepared F5 from cultures grown in 2% raffinose medium for 24 h. Sufficient adenine was added to allow normal growth and prevent a selection for enhanced *ADE2* expression. The uninduced *ADE2* mRNA levels were comparable and reduced about 10 times for the p53-responsive promoters compared to the natural *ADE2* promoter. The actin mRNA levels were similar for all cultures.

The levels of mRNA induced by p53 at various REs reflected colony color. p53-dependent *ADE2* transactivation was measured with low (raffinose) and high (galactose) p53 expression. Total RNA was prepared from liquid raffinose cultures (i.e., basal level of induction) of yAFM-RE strains that produced red (RGC, PCNA, MDM2-RE1, BAX-A, cyclin G, and AIP1), pink (GADD45), and white (P21-5′ and p53-R2) colonies on this medium (Fig. 5B). Consistent with the phenotypic assay, the *ADE2/ACT1* ratio was about five times greater for the two white strains than for the red strains. However, the pink yAFM-GADD45 strain was not distinguishable from the red strains.

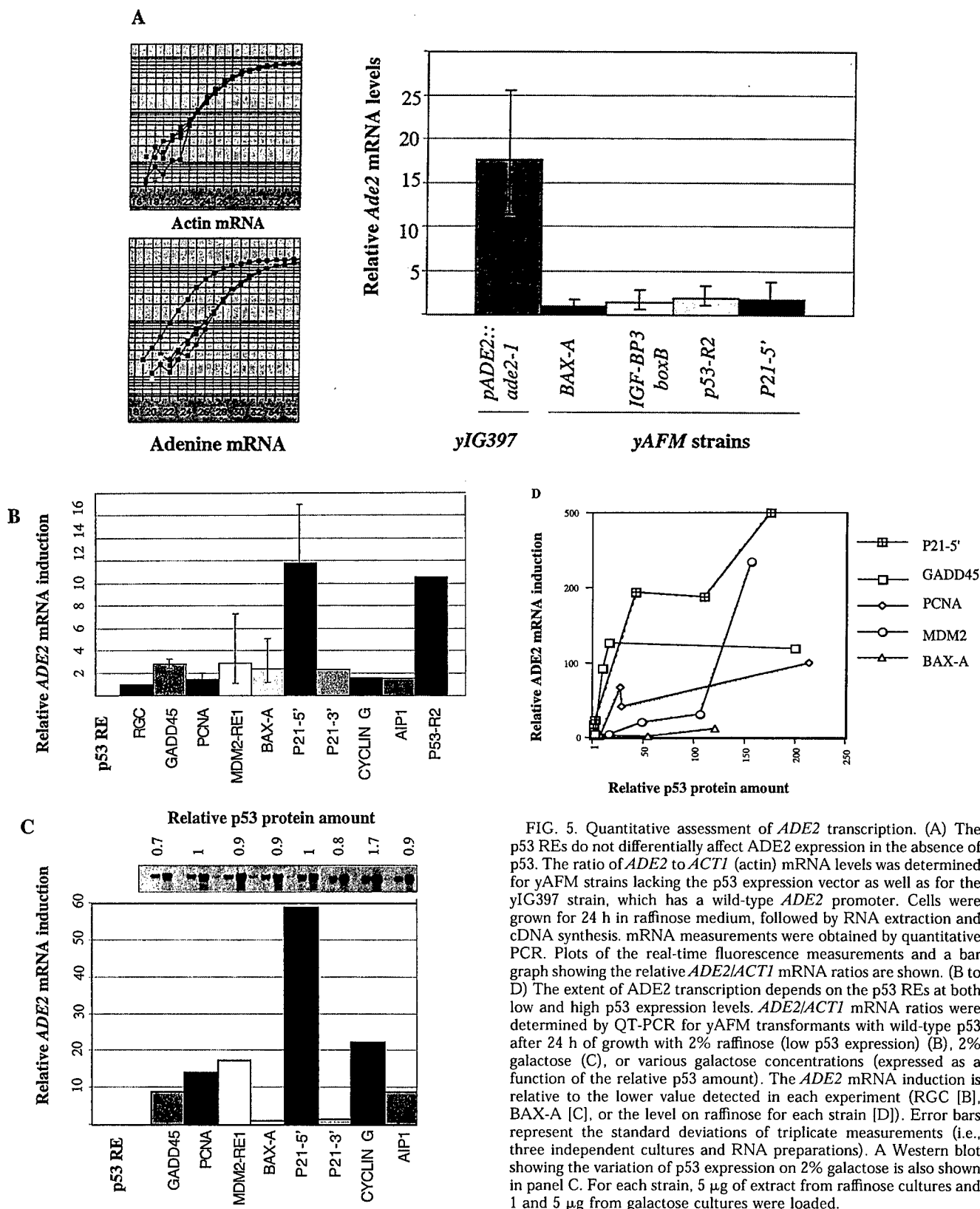RNA was also examined following growth in 2% galactose

FIG. 5. Quantitative assessment of *ADE2* transcription. (A) The p53 REs do not differentially affect ADE2 expression in the absence of p53. The ratio of *ADE2* to *ACT1* (actin) mRNA levels was determined for yAFM strains lacking the p53 expression vector as well as for the yIG397 strain, which has a wild-type *ADE2* promoter. Cells were grown for 24 h in raffinose medium, followed by RNA extraction and cDNA synthesis. mRNA measurements were obtained by quantitative PCR. Plots of the real-time fluorescence measurements and a bar graph showing the relative *ADE2/ACT1* mRNA ratios are shown. (B to D) The extent of ADE2 transcription depends on the p53 REs at both low and high p53 expression levels. *ADE2/ACT1* mRNA ratios were determined by QT-PCR for yAFM transformants with wild-type p53 after 24 h of growth with 2% raffinose (low p53 expression) (B), 2% galactose (C), or various galactose concentrations (expressed as a function of the relative p53 amount). The *ADE2* mRNA induction is relative to the lower value detected in each experiment (RGC [B], BAX-A [C], or the level on raffinose for each strain [D]). Error bars represent the standard deviations of triplicate measurements (i.e., three independent cultures and RNA preparations). A Western blot showing the variation of p53 expression on 2% galactose is also shown in panel C. For each strain, 5 μg of extract from raffinose cultures and 1 and 5 μg from galactose cultures were loaded.

for all but the RGC and p53R2 strains. As shown in Fig. 5C, the BAX-A strain, which is pink on 2% galactose plates (Fig. 4), showed the lowest *ADE2* induction. All of the other strains were white on 2% galactose. However, there were differences in mRNA induction that ranged from 1.5 times higher for the yAFM-P21-3′ strain to 60 times higher for the P21-5′ strain compared to levels in the BAX-A strain. The other strains showed between 10 and 20 times higher levels of *ADE2* mRNA. Since the p53 protein levels of cells grown on 2% galactose were comparable, the differences in expression must be due to differences in transactivation capacity (Fig. 5C).

Finally, we examined the relative *ADE2* transcription from the P21-5′, GADD45, PCNA, MDM2-RE1, and BAX-A REs at different levels of p53 induction. Total RNA was prepared from the same cultures used for the Western blots presented in Fig. 2A, and the *ADE2/ACT1* ratios are presented in Fig. 5D. The yAFM-BAX-A strain exhibited poor induction at all levels of p53. The P21-5′, GADD45, and PCNA strains had various degrees of strong induction of *ADE2* at relatively low levels of p53 protein. Except for P21-5′, the induction of transactivation reached a plateau at high levels of p53 protein expression. The yAFM-MDM2-RE1 strain showed a modest stimulation of *ADE2* transactivation up to a 100-fold increase in p53. This RE becomes highly responsive only with large amounts of p53 (i.e., 2% galactose). We conclude that there is a good correlation between the phenotypic assay and transactivation of the *ADE2* gene.

The CATG sequence at the center of a p53 dimer binding site greatly affects RE activity. We noticed that the 5′-CATG sequence at the junction between p53 monomer binding sites is found in both dimer sequences of the four strongest REs described in Table 1: P21-5′, p53R2, m-FAS, and GADD45. This motif is present in only one dimer sequence in five other REs in Table 1. To evaluate the contribution of this sequence element to p53 transactivation capacity, we examined 4 additional artificial sequences, described in Table 1 as Con-A, Con-B, Con-C and Mut-RGC. The Con sequences correspond to the consensus RRRCWWGYYY. Interestingly, Con-A, which contains the CATG sequence in both dimer elements, showed the strongest activity of all of the REs, as shown in Fig. 4 (the colonies were white on glucose plates). Con-B, which is identical to Con-A but has CTAG central elements, ranked sixth, with an approximate 20-fold reduction in transactivation capacity. There was less transactivation from Con-C (it ranked eighth), possibly because it lacks any CATG element. We also constructed a modified RGC RE (Mut-RGC), replacing the CTTG sequence element with CATG in both dimers. This RE showed a dramatic increase in activity.

Subtle changes in transactivation capacity are revealed in p53 mutations associated with familial breast cancer. The incidence of p53 mutations in familial breast cancer associated with germ line BRCA1/2 mutations is nearly 70%, compared to around 30% for sporadic breast cancer. Moreover, the spectrum of mutations appears to be different (30). Interestingly, a subset of BRCA1-associated p53 mutant alleles appeared wild type in mammalian functional assays (67), and four of the mutants, T150I, G199R, R202S, and S215C, were also wild type in a yeast transactivation assay at high p53 expression (11). We tested these mutants for the ability to transactivate from 10 of the REs in this study at various levels of p53

TABLE 2. Relative transactivation capacity at low levels of expression of p53 mutant alleles associated with familial breast cancer[a]

RE	Transactivation capacity[b] with p53 mutant allele:			
	T150I	G199R	R202S	S215C
P21-5′	wt	L	wt	wt
p53 R2	L	L	L	L
GADD45	wt	L	L	H
hFAS	wt	L	L	wt
AIP1	wt	H	L	H
PCNA	wt	L	L	H
Cyclin G	L	H	L	wt
NOXA	wt	wt	L	wt
PUMA	wt	L	wt	H
BAX-B	wt	H	L	H

[a] There is no difference at high levels of expression.
[b] wt, wild-type activity; L and H, two- to fourfold lower or higher transactivation capacity, respectively, than the wild type.

expression. All of the mutants were comparable to the wild type at high levels of p53 expression (data not shown); however, the matrix of responses differed from wild type at low levels. The difference in the minimal galactose amount needed to obtain white colonies (or light pink colonies with BAX-B) with mutant and wild-type alleles was used to estimate the relative transactivation capacity. Subtle differences in the transactivation pattern were detected for all four alleles (Table 2). Interestingly, the changes comprised both reduced and enhanced activity with specific REs and no effect with other REs relative to the wild type, suggesting that the amino acid changes do not simply affect protein stability.

DISCUSSION

What is the role of p53 intrinsic DNA binding capacity in differential transactivation? Since differential modulation of transcription of many genes by a common regulatory protein can provide for adaptive cellular responses, particularly in response to environmental stress, it is important to understand the underlying mechanisms. There are many factors that can influence the specificity, levels, and kinetics of transcriptional changes of various genes. As described in Fig. 6 they include the amount and activity of the transactivation protein (e.g., p53), the availability of cofactors, the chromatin organization of the gene locus, and the actual sequence of the target promoter elements. A minimal functional unit that is required, but is not sufficient, for transcriptional activation is the transcription factor bound to DNA. The specific DNA binding activity of factors such as p53 may facilitate the recruitment of chromatin-remodeling systems that in turn modulate the efficacy of the assembly of the transcriptional machinery and of transcription initiation and elongation (17, 59).

A fundamental question that has not been adequately addressed with in vivo systems concerns the role of intrinsic DNA binding specificity in differential transactivation (5, 47, 61). A common feature of several families of response elements is variation in nucleotide sequence, reflected by a loose consensus within a family (51, 60). Hence, the relative binding affinity or transactivation potential of specific promoter elements by a common regulator might allow for flexibility in cellular re-

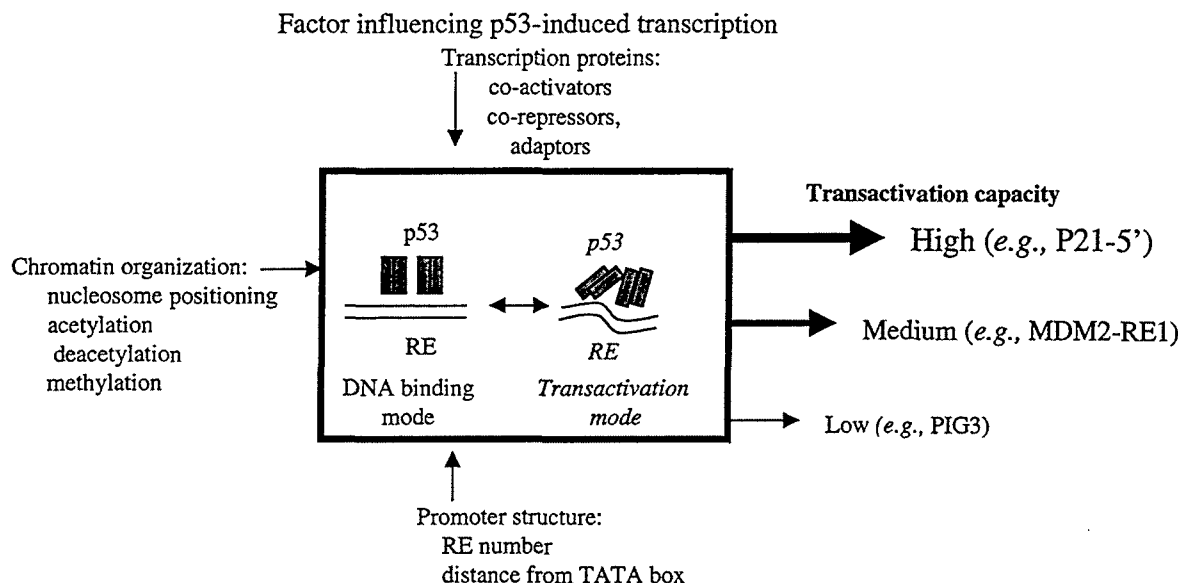Factor influencing p53-induced transcription

FIG. 6. Intrinsic DNA binding and differential transactivation by p53. Many factors can influence p53 transactivation (arrows). In this study, the factors are kept constant by using isogenic yeast in the functional assay. Thus, a difference in transactivation capacity solely reflects differences in the intrinsic DNA binding affinity of p53 for the individual REs (box). Following sequence-specific DNA binding, p53 may undergo conformational changes that alter tetramer stability and favor protein-protein interaction with the basal transcriptional machinery.

sponses. This is particularly relevant in the case of the p53 transcription factor, whose nuclear levels increase in response to cellular stresses and which can directly affect transcription of many genes with different functions. It is also relevant in light of the many mutant p53 alleles selected and expressed in tumor cells that appear to retain partial activity (11, 28, 67) (R. Storici et al., unpublished data).

Based with systems that utilize high constitutive p53 expression, there is an abundance of anecdotal evidence of differential transactivation capability in mammalian cells and in yeast by wild-type p53 or p53 mutants (18, 62, 72). Genome expression analyses have shown that there are large differences in transactivation (and repression) of the many p53 target genes in response to different levels of ectopically expressed wild-type p53 or in response to the induction of endogenous p53 by various sources of DNA damage (40, 41, 64, 88). However, the source and degree of differences have not been clear.

Most of the studies addressing p53 binding to REs and its regulation, which are based on in vitro experiments with naked DNA, are supportive of a general model whereby p53 in unstressed cells is a latent DNA binding protein that requires posttranslational modification for activation (35, 62, 71). However, these assays may not accurately mimic the nuclear environment. Recent in vivo studies that utilize chromatin immunoprecipitation have shown variation in promoter occupancy by a constitutively active p53 protein in unstressed cells at a small number of targets. Induction of p53 by genotoxic stress led to an increase in DNA binding that correlated with the increase in protein amount with no evidence of changes in affinity (39, 69). However, the correlation between level of promoter occupancy and strength of transactivation by p53 was not precise and is likely influenced by specific features of the target loci. With this in mind, we sought to develop a sensitive in vivo system that could address the importance of both p53

levels and RE sequence in differential transactivation. To do this, we kept constant all other potentially modifying factors (Fig. 6), such as number of REs, chromatin structure, coregulators, and p53 posttranslational modifications, by constructing a panel of isogenic yeast strains, each containing a p53-dependent reporter gene, that differ only in the sequence of the p53 RE.

RE sequence and p53 protein level play an important role in differential transactivation. Using our model system, we have established that both the RE sequence and the levels of p53 induction are important factors in determining which RE is activated and to what extent. The wide linear range of protein expression (Fig. 2) allowed us to distinguish both subtle and dramatic differences in relative p53 transactivation capacities among 26 p53 REs. The difference in p53 protein amount required to achieve the same transactivation with two REs provided a measure of relative transactivation capacity. This highly reproducible phenotypic assay for p53 transactivation function was validated by mRNA measurements. Since we used isogenic strains and conditions, the transactivation differences directly relate to DNA binding of p53 with its REs.

Our results support the dose-response model of p53 regulation in mammalian systems (44), in which various biological responses are dependent on the level of available nuclear p53. We could not correlate transactivation capacity with gene groupings in functional pathways. However, the weak transactivation group is enriched with REs from apoptotic genes, which is consistent with results for mammalian cells, where induction of the apopotic pathway appears to have a higher threshold of activation (15, 63, 78). Also, the weak transactivation potentials of MDM2-RE1 and -RE2 are consistent with the important role of this gene as a p53 repressor, and they parallel results obtained with mammalian cells (31, 45, 74).

The wide differences in transactivation capacity, as much as

Q: G

TABLE 3. Relative p53 binding or transactivation capacity towards various REs: comparison of transactivation in yeast
with reported in vitro EMSA or ChIP results from human cells

Assay	Relative (%) p53 transactivation capacity or p53 binding to the following RE[a]:									Reference
	P21-5'	PUMA	BAX-A + B	MDM2-RE1 and -RE2	NOXA	P21-3'	BAX-B	BAX-A	PIG3	
Yeast transactivation	100	~30	~30	~15	~15	~10	≤2[b]	≤0.1[b]	≤0.1[b]	This study (Fig. 4)[c]
EMSA	100		~10				≤3[d]	≤3[d]		71 (Fig. 4)
EMSA	100					~30				62 (Fig. 4)
EMSA	100			~100[e]					≤1[d]	69 (Fig. 6)
ChIP[f]	100	~100	~15	~200	~15				~5	39 (Fig. 1)

[a] The strongest element in the yeast assay is given the value of 100%, and responses with other elements are compared with it.

[b] Colonies do not turn white even at the highest p53 expression level; they remain light pink (BAX-B) or pink (BAX-A and PIG3).

[c] The amount of p53 protein required for the appearance of white colonies with the different REs has been used to measure relative transactivation capacity.

[d] Based on relative amounts of REs to achieve equal competition. In the case of BAX-A, BAX-B, and PIG3, this is the largest amount tested, and it appears that the maximum capacity to compete is reached.

[e] Two shifted bands were detected, since this RE contains two separate p53 tetramer binding sites. As a competitor, the P21-5' RE was more efficient than MDM2-P2 at displacing one of the two p53 tetramers but not at completely displacing bound p53 proteins.

[f] The natural promoters from the human H1299 cell line have been probed, and hence a comparison of RE affinity in this analysis can be influenced by specific chromatin structure in the promoter region.

1,000-fold, were unexpected. The patterns could be divided into three groups. The strongly binding group, where white colonies could be detected at low levels of galactose, includes the REs from Con-A to cyclin G in Table 1 and Fig. 4 (strongest to weakest). Presumably, p53 tetramers can efficiently form at these sites and lead to transcription. The next group, from NOXA to MDM2-RE2, showed modest induction over a broad range of p53 expression. For the yAFM-MDM2-RE1 strain, comparable levels of *ADE2* mRNA were detected over a 100-fold induction of p53, and strong induction was observed only on 2% galactose. Six REs, from cFOS to IGF-BP3 box B, led to poor transactivation even at the highest levels of p53 (Fig. 4). This suggests that factors other than RE sequence, such as chromatin state, distance to or from the transcription start site, and availability of coactivators, might be important for transactivation from these promoters in mammalian cells (65). These REs may be ideal for identifying such factors in a yeast-based system. In addition, efficient transactivation of a subset of target genes by p53 may require the function of the p53-related p63 and p73 genes (26). Interestingly, in mouse embryo fibroblasts such cooperation appeared to be specifically required for the induction of a subset of p53 targets whose REs scored as intermediate or weak in our yeast-based functional rank (BAX and NOXA) (Fig. 4) (26). We are currently evaluating the transactivation capacities of p63 and p73 isoforms and the effect of their coexpression on p53 function by using the functional assay at low or variable expression levels (unpublished results).

To better compare the relative activities, we chose to focus this study on p53 REs containing a single tetramer binding site (with the exception of cFOS), even for those cases of human genes that have more than one RE. Perhaps more than one RE is required for transactivation of genes that contain a poorly responding RE. The presence of two closely spaced REs, as found, for example, in the human *mdm2*, *bax*, and *IGF-BP3* genes, could result in a cooperative effect to make the promoter responsive to p53. (The results with cFOS do not contradict this view, since transcription from just one cFOS tetramer was not examined.) In a separate study (unpublished), we examined transactivation from the MDM2-P2 sequence (where the two REs are separated by 5 nucleotides) and from

BAX-A+B, IGF-BP3 box A+B, and 2× RGC containing two contiguous copies of the RE without spacer. Unlike the situations with a single RE, these strains turned white with modest to high levels of p53 expression, supporting the idea that adjacent REs can have a cooperative effect on transactivation capacity. BAX-A+B showed a profile of activity similar to that of cyclin G (11th in the ranking), while the other three strains were similar to NOXA (data not shown). However, the transactivation capacities of these complex REs were still significantly lower than those of the top-ranked single REs such as P21-5', p53-R2, and FAS (Fig. 4).

Overall, the yeast-based results are consistent with electrophoretic mobility shift assays (EMSA) and chromatin immunoprecipitation (ChIP) analysis (e.g.P21-5', P21-3' BAX-A, BAX-B, MDM2, NOXA, PUMA, and PIG3) (39, 62, 69, 71, 82). The yeast-based ranking of transactivation capacities is also consistent with the limited gene expression studies that are available for comparison (e.g., p21, *Gadd45*, *Pig3*, and *bax*) (25, 40, 69, 88). Comparisons between the yeast results and published DNA binding measurements for nine REs are presented in Table 3. There is a good qualitative correlation between relative in vitro binding activity and transactivation capacity. The correlation is less apparent for comparisons with ChIP results. However, this in vivo assay is not well suited to evaluate relative DNA binding capacities, since each RE is analyzed within different promoters where individual differences in chromatin structure are expected to influence p53 occupancy. The large difference in transactivation capacity between the two p21 REs in yeast is also consistent with analysis of in vitro DNA binding to chromatin, although there was only a small difference in p53 binding to the two sequences (23). Similar to results from that chromatin study, we also observed that the carboxy-terminal deletion (Δ368-393) leads to a reduction, rather than stimulation, of p53 transactivation capacities with all REs, especially for the weaker REs that include P21-3' (unpublished results).

We have also applied our analysis at low or variable p53 expression levels to the evaluation of the role of the two distinct transactivation (TA) domains located in the p53 amino terminus. Deletion of the first TA domain (Δ1-39) resulted in an approximately 50% reduction in transactivation capacity

TABLE 4. Nucleotide usage derived from a panel of p53 REs previously identified (73) in a transactivation assay in yeast

Nucleotide	Usage (%) for the following position in the consensus sequence at the p53 dimer binding site:									
	R	R	R	C	W	W	G	Y	Y	Y
A	26.9	29.1	34.7	1.7	77.1	19.3	1.1	14.6	12.3	12.5
G	55.4	55.2	54.9	0.0	2.9	21.1	97.7	5.3	19.3	4.8
C	7.4	2.9	2.9	98.3	2.9	0.6	0.0	28.7	45.6	50.0
T	10.3	12.8	7.5	0.0	17.1	59.1	1.1	51.5	22.8	32.7

with all 12 REs that were tested (unpublished results). With strong REs like P21-5', p53R2, and GADD45, white colonies were obtained with moderate p53 expression. At high p53 expression, a defect in transactivation was visible only with intermediate and weak REs such as P21-3', MDM2-RE1, and PA26. Consistent with this observation, a previous analysis at high p53 expression of a double mutant in the first TA domain (L22E/W23S) showed almost no affect on transactivation function, pointing to differences in the interactions with the transcriptional machinery between yeast and mammalian cells (18). We also tested single amino acid changes (W53R and F54S) at key residues in the second TA domain (12) and observed a reduction in transactivation capacity similar to that seen with the Δ1-39 mutant. Deletion of both TA domains (Δ1-65) instead completely abolished p53 transactivation capacity. These combined observations suggest that the TA domains have overlapping functions in yeast and contribute comparably to total protein transactivation activity, a feature that could be appreciated only at low or variable p53 expression and that is consistent with observations for human cells (89).

The posttranslational modification status of p53 protein expressed in yeast has not been reported. Hence, we cannot exclude the possibility that the functional rank we described results from a particular state of p53. In mammalian cells, it is not clear whether p53 posttranslational modifications play any direct role in p53 DNA binding specificity (1, 6, 19). It is important to note that neither an *MDM2* homologue nor a p53 homologue has been detected in budding yeast. The transactivation capacity of p53 alleles with amino acid changes at sites of posttranslational modifications when expressed alone or in combination with the MDM2 protein is currently under investigation.

Understanding differences in p53 transactivation capacity. We sought to explain how the small differences in 20 bp of the REs could account for the vast differences in transactivation capacity. The number of nonconsensus bases in the RE sequences (Table 1) clearly does not predict the wide variations. For example, both P21-5'and IGF-BP3 box A have two nonconsensus bases at the first position of monomer binding sites, and they display a dramatic difference in transactivation.

A statistical method based on nucleotide usage has been developed to predict the relative activity of each RE (4). We computed the nucleotide usage (Table 4) of a large panel of p53 REs previously identified by Tokino et al. (73) by using yeast-based transactivation at high p53 expression (T values). A total of 170 p53 dimer REs were reported in that study. We also used the published nucleotide usage obtained with 20 REs identified by in vitro binding assays (B values) (21). The nu-

cleotide usage value (percent) for each position was used to compute the heterology index (HI) of each RE with an equation derived from statistical mechanics theory (4) (Table 1). Although some of the more active REs have lower HIs, particularly when the T values of nucleotide usage are used, we found that nucleotide usage approaches have low predictive value. For example the most active P21-5' RE has only the 9th and 10th best HIs, respectively, with the T and B nucleotide usage values. Moreover, RGC is predicted to have a similar or higher activity than P21-5', despite about a 1,000-fold difference in transactivation capacity. The statistical approach is based on the assumption of equal contributions of each binding unit (i.e., each nucleotide in the RE) to the affinity or activity of the complete RE. No additional correction factor is introduced to weight the position of each nucleotide within a p53 monomer binding site. An alternative approach would involve weighting the contribution of nucleotides directly involved in protein-DNA interactions, i.e., positions 3 and 4, as predicted by the crystal structure (16). Even though the functional rank is partially consistent with a limited role of the nucleotide at position 1 of the p53 monomer binding site and a higher impact of deviations at position 3 (e.g., RGC and Mut-RGC) (Table 1), the overall result cannot be completely explained by the position of the nucleotide that deviates from the consensus. For example the RE used in the crystal structure study (Con-C) has a perfect consensus sequence but ranked eighth in our transactivation assay, and the PCNA RE with a mismatch at the invariant fourth position still retains strong transactivation capacity.

Another interesting algorithm that identifies putative p53 REs and computes their binding probabilities by using a novel filtering and weight matrix was recently reported (33). No correlation was found with results in the present study. The algorithm attributed the same binding probabilities to IGF-BP3 A and H-FAS (scored as 90.9 and 90.92 out of 100), while the yeast-based functional assays indicated almost a 1,000-fold difference in transactivation capacity. MDM2-RE1 had a much lower score (79.68), while the order in the yeast assays was H-FAS, MDM2-RE1, and IGF-BP3 A (Fig. 4).

We hypothesize that sequence-dependent structural features of the DNA greatly affect p53 activity in our in vivo system, as previously observed in vitro with purified p53 DNA binding domain and naked DNA RE (54). It is possible that upon interaction with DNA RE sequences the p53-DNA complex undergoes conformational changes that significantly contribute to binding affinity and activity. For example, axial bending of the DNA facilitated by the flexibility of the CATG sequence (55) may lead to tighter p53 tetramer binding by allowing the establishment of intersubunit interactions in addition to those at the tetramerization domain (50). These changes might also make available the amino-terminal transactivation domains for protein-protein interactions with components of the transcription machinery. Our results with the consensus sequences and the modified RGC RE support this interpretation. Modification of Con-A (CATG) sequences into Con-B (CTAG) (Table 1) led to a considerable reduction in activity. Furthermore, modification of the RGC RE (CTTG into CATG, corresponding to Mut-RGC) resulted in a dramatic improvement of p53 transactivation capacity. The observation that subtle changes in RE sequence can lead to signif-

icant differences in the responsiveness of a target gene to p53 also suggests that interspecies, interindividual, or somatically acquired variations in RE sequences may lead to diverse p53-dependent responses, which has implications for cancer susceptibility and evolution.

Effects of p53 tumor and other mutations on differential transactivation. The yeast functional assay that we described provides a versatile tool to evaluate the impact of p53 mutations on transactivation potentials and specificity. One implication of the important role of p53 intrinsic DNA binding affinity in differential transactivation is that specific p53 mutations may lead to defined changes in the transactivation patterns that are compatible or advantageous during tumorigenesis and could be selected in particular cellular or genetic environments. The rheostatable system along with many REs enables the functionality of various p53 mutations to be addressed.

Previously we found several alleles that were toxic at high expression levels but exhibited increased transactivation toward the P21-5' RE at basal-level expression (37). The functional profile of these alleles with the complete panel of REs is being determined. The system might provide the opportunity to select p53 alleles that would have altered functional patterns in mammalian cells, such as apoptosis (39, 64). For example, a V122A mutant shows a 4- to 8-fold higher transactivation capacity toward P21-5', GADD45, and hFAS and about a 16-fold higher capacity toward BAX-A+B and IGF-BP3 box A (unpublished results). Its activity was equal to that of wild-type p53 with p53R2, while it was reduced four- to eightfold with 14-3-3 and cyclin G and was undetectable with P21-3', MDM2-RE1, and RGC (unpublished results).

The p53 mutant alleles detected in familial BRCA1/2-associated breast cancer are an important example of how the opportunity to address transactivation capacity over a wide range of p53 levels can be useful in understanding functional alterations. We analyzed four alleles that had been characterized previously in both mammalian and yeast assays at high levels of expression. They appeared to be indistinguishable from wild-type p53 (11, 67) in a number of in vivo functional assays that were used. As shown in Table 3, the yeast assays at low and variable p53 expression provide for functional discrimination. While the differences were small (about two- to fourfold) and a common pattern of functional changes was not evident, both reductions and increases in transactivation capacity with defined REs were observed. These results may implicate subtle changes as having an important impact on the etiology of tumor development.

These observations with wild-type p53 and mutant alleles have general implications for studies with other families of transcription factors whose nuclear levels are tightly controlled and which recognize response elements that have loose consensus sequences, as for the case of p53. The system of tight regulation of protein expression, rapid construction of responsive promoters by using in vivo delitto perfetto site-directed mutagenesis (68), and phenotypic and quantitative assessment of transcription levels provides a framework in which to investigate a variety of issues that relate to transactivation specificity. The approach provides the means to study altered selectivity and differential transactivation in vivo as well as the isolation and characterization of enhancer elements and transcription factors and the screening for external modifiers of transcription complex assembly.

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APPENDIX 2

Figure 1. The tumor suppressor p53 is a sequence-specific transcription factor.

Many types of stress induce signal transduction pathways that lead to p53 stabilization

and activation, mainly by post-translational modifications. The p53 protein is shown as a

tetrameric (dimer of dimers) sequence-specific transcription factor that recognizes

promoter Response Elements. The solid arrows indicate the organization of the RE

sequence as a closely-spaced pair of dimer-binding sites each consisting of two

monomer-binding sites in inverted orientation. A list of the principal p53 target genes and

their functions is shown.

Figure 2. p53 structural organization, topological domains and location of tumor

mutations. [a] Functional domains. TA I and II = acidic transactivation domains; amino

acids 22 and 23 are required for TA I while 53 and 54 are crucial for TA II. One nuclear

export signal NES whose activity is influenced by the phosphorylation status of p53

overlaps with TA I. PxxP = proline-rich domain with possible role in protein:protein

interactions (20). TD = tetramerization domain; NLS = nuclear localization signal; RR=
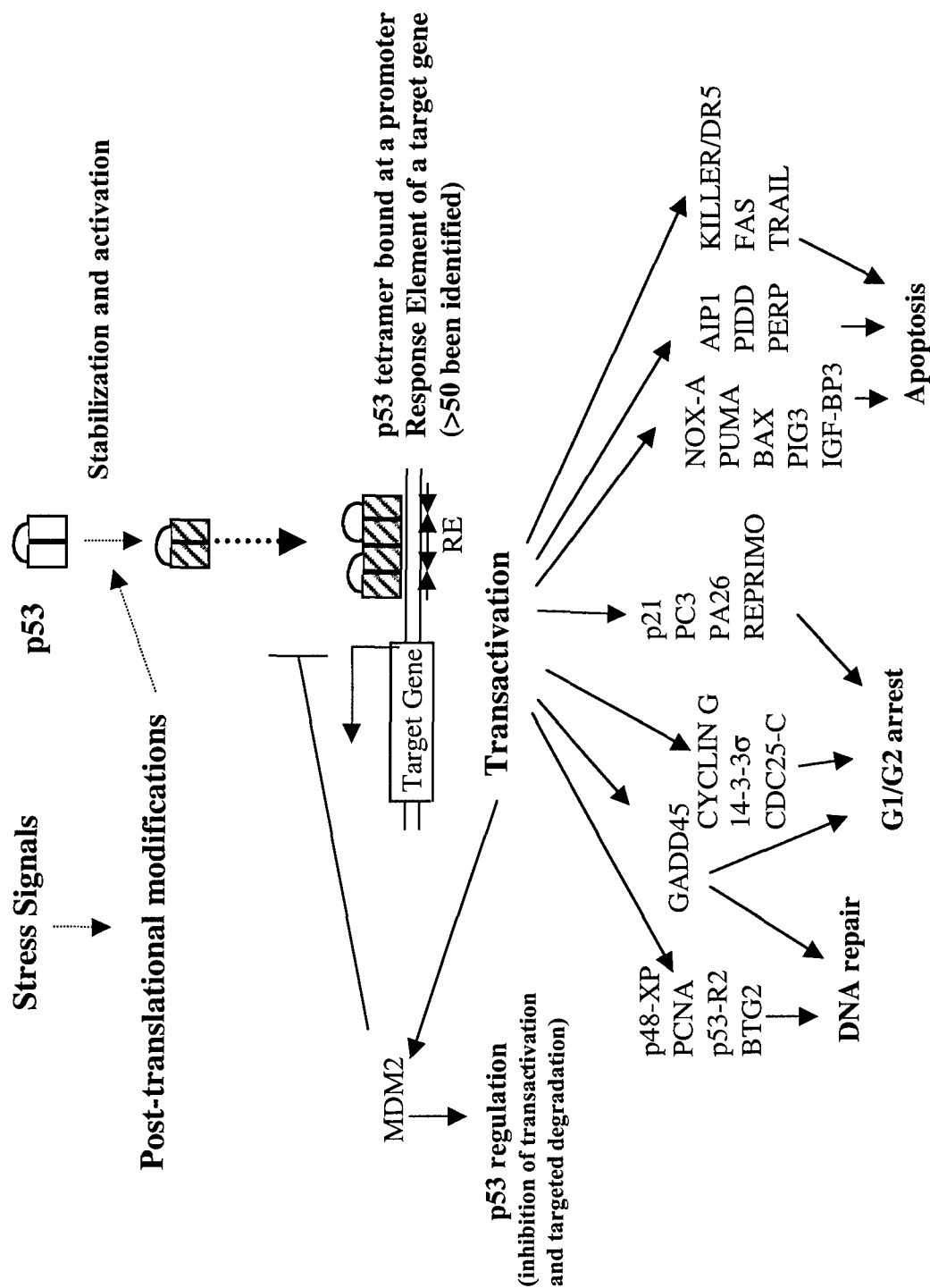
basic regulatory region.

[b] Conserved domains. The positions of the 5 evolutionary-conserved regions along the

393 p53 amino acid sequence are indicated. 4 of them correspond to sequences within the

sequence-specific DNA binding domain. With the exception of domains I and II, the

major p53 hotspots are located in the conserved domains. Presented is the distribution of

the tumor p53 mutations along the protein (hotspots are indicated by the number in the

graph). Data from the R5 release of the IARC p53 mutation database (containing more

than 15,000 mutations; http://www.iarc.fr/P53/Somatic.html) were used to generate the

graph.

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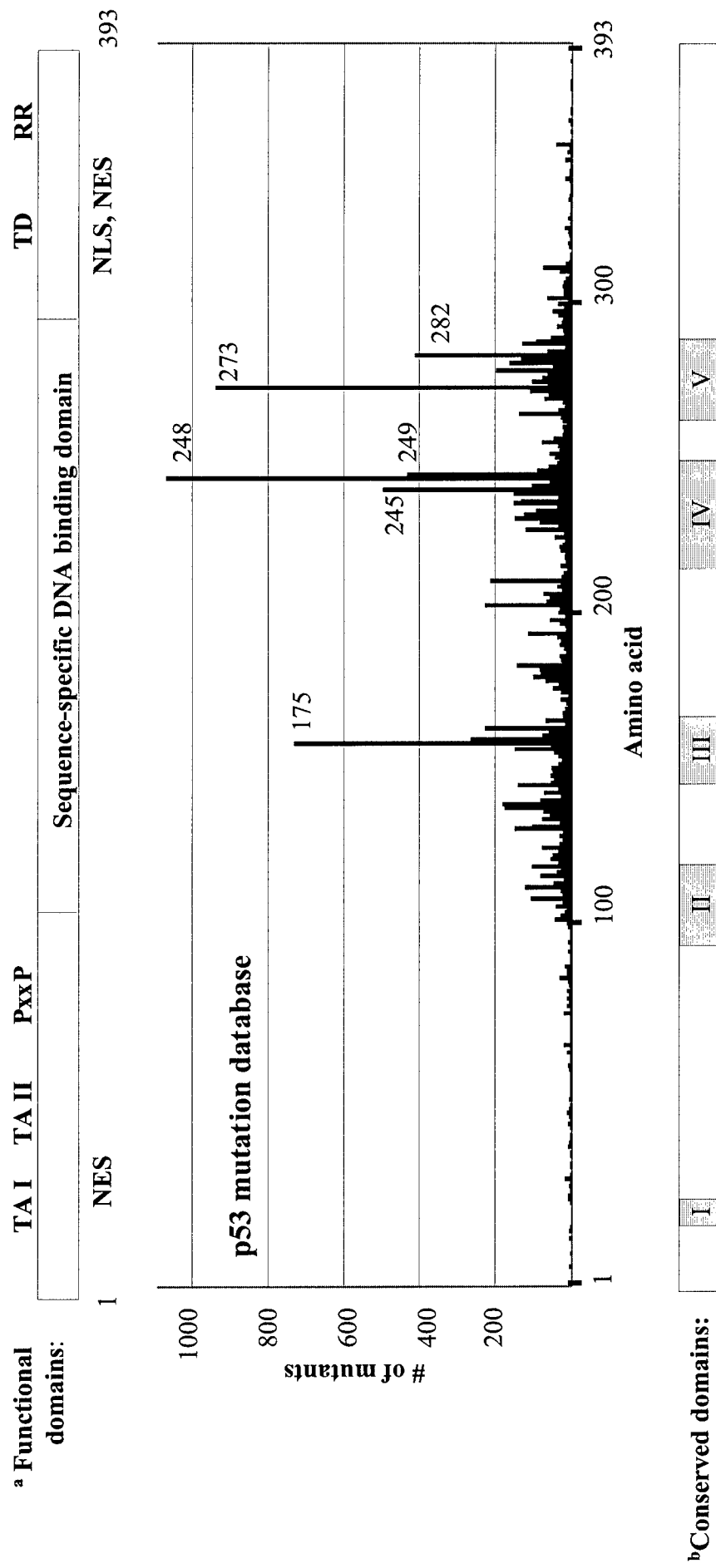**Figure 3. General features of the p53 functional assay in yeast.**

A. Expression of p53 in yeast and transactivation by p53. The complete cDNA of a given

p53 allele can be expressed under either a constitutive or an inducible promoter from a

selectable plasmid. The centromeric plasmids are stably transmitted at low copy number.

WT p53 can act as a transcription factor in yeast stimulating the activity of a promoter

whose upstream activating sequences have been replaced with a p53 response element.

Mutant p53 would change the level of transcription of the reporter gene thus leading to

phenotypic changes. If the p53 is not active, no transactivation is observed. If the p53

allele is less active, higher levels of *GAL1* induction are needed; if more active, less

induction is required for a phenotypic change.

B. Summary of reporter genes and the assays used for phenotypic analysis of p53 mutant
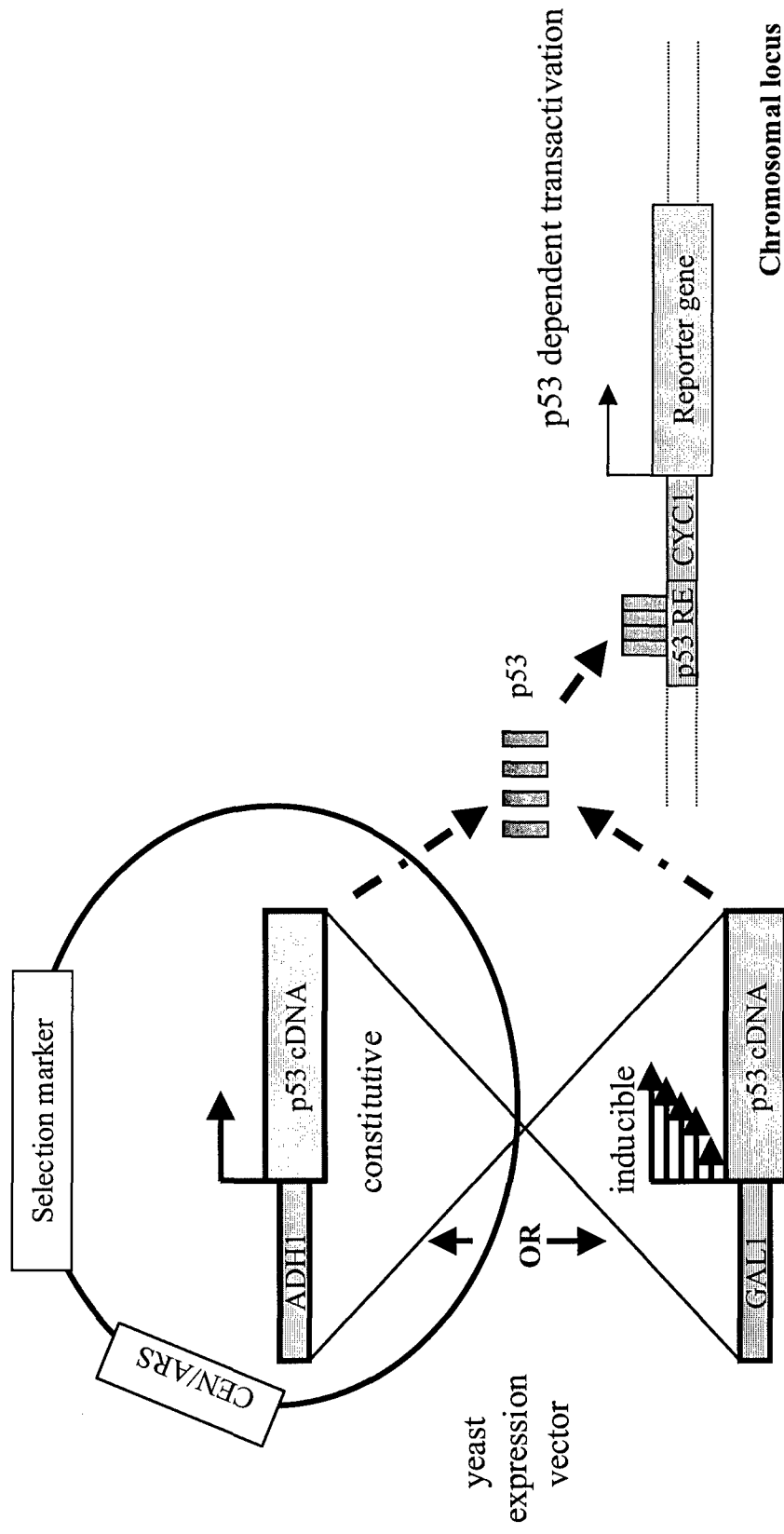
alleles under conditions of high expression.

C. Example of phenotypic assays using the *ADE2* red/white reporter gene. Under

conditions of high constitutive expression of WT p53, transactivation of *ADE2* results in

white colonies. Loss-of-function p53 mutants will result in red colonies since they cannot

turn on *ADE2*. When the *GAL1*-based inducible expression system is used,

transactivation is proportional to the levels of WT (or mutant) p53 protein expressed.

Presented are yeast transformants with WT p53 grown on plates containing different

amounts of galactose inducer (plus raffinose carbon source). Also shown are western

blots of protein extracts from cells grown under the same conditions. The appearance of

colonies extends from red to pink to white as the amount of p53 is increased.

ᵃ Functional domains:

TA I   TA II   PxxP   TD   RR

| NES | Sequence-specific DNA binding domain | NLS, NES |

1   393

p53 mutation database

# of mutants

1000
800
600
400
200

175
248
273
282
245
249

100   200   300   393

Amino acid

ᵇConserved domains:

I   II   III   IV   V

p53 functional assay in yeast: transactivation of a reporter gene containing a p53 response element upstream of a minimal promoter.
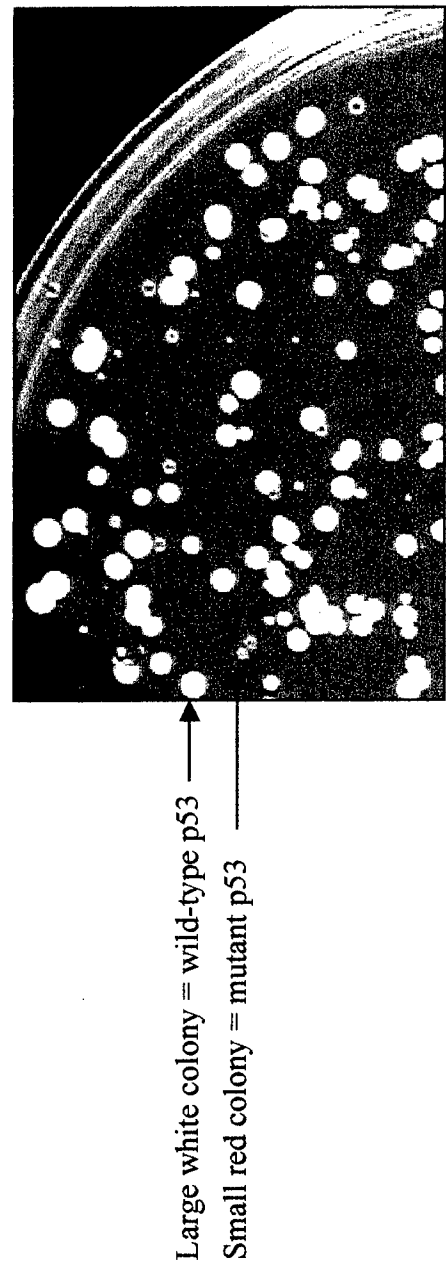
Phenotypic characterization of p53 null alleles based on transactivation of a reporter gene
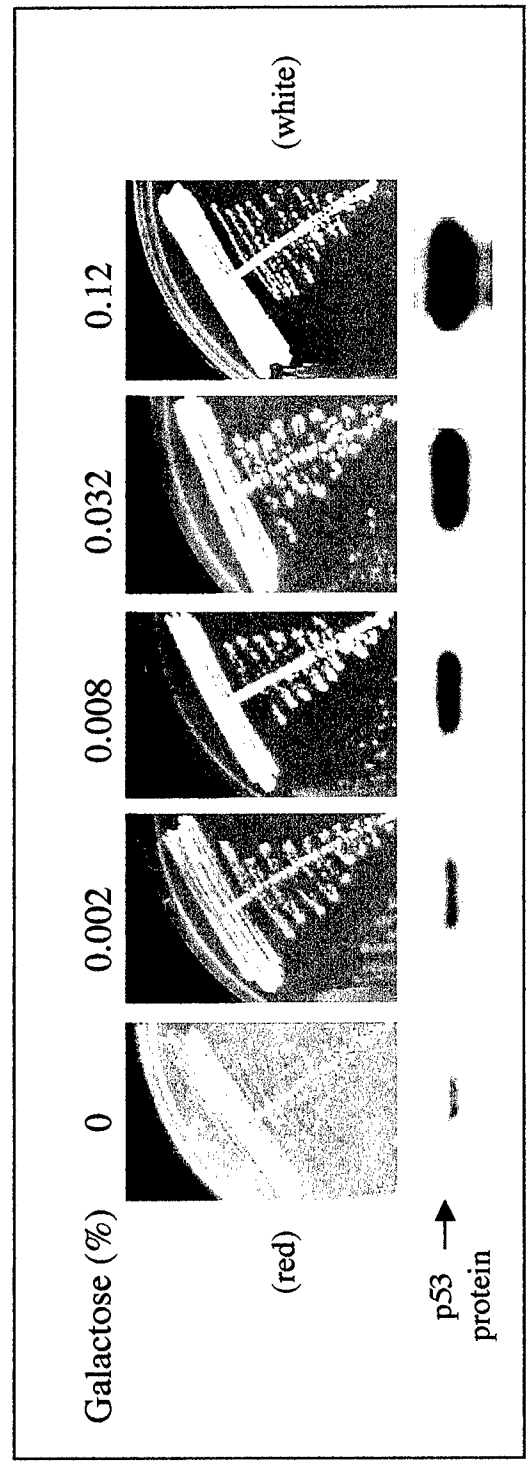
	Reporter gene:		
	HIS3	*URA3*	*ADE2*
Assay:	growth on his⁻	growth on ura⁻ or 5-FOA	growth on plates with low adenine
Response:			
Wild type p53:	growth	growth on ura⁻ and 5-FOA sensitive	growth, white color
p53 mutant:	no growth	no growth on ura⁻ and growth on 5-FOA	slow growth, red color

Phenotype of wild-type and mutant p53-expressing cells using the *ADE2* reporter gene

A. Constitutive p53 expression under the *ADH1* promoter

Large white colony = wild-type p53
Small red colony = mutant p53

B. Inducible expression of wild-type p53 under the rheostatable *GAL1* promoter

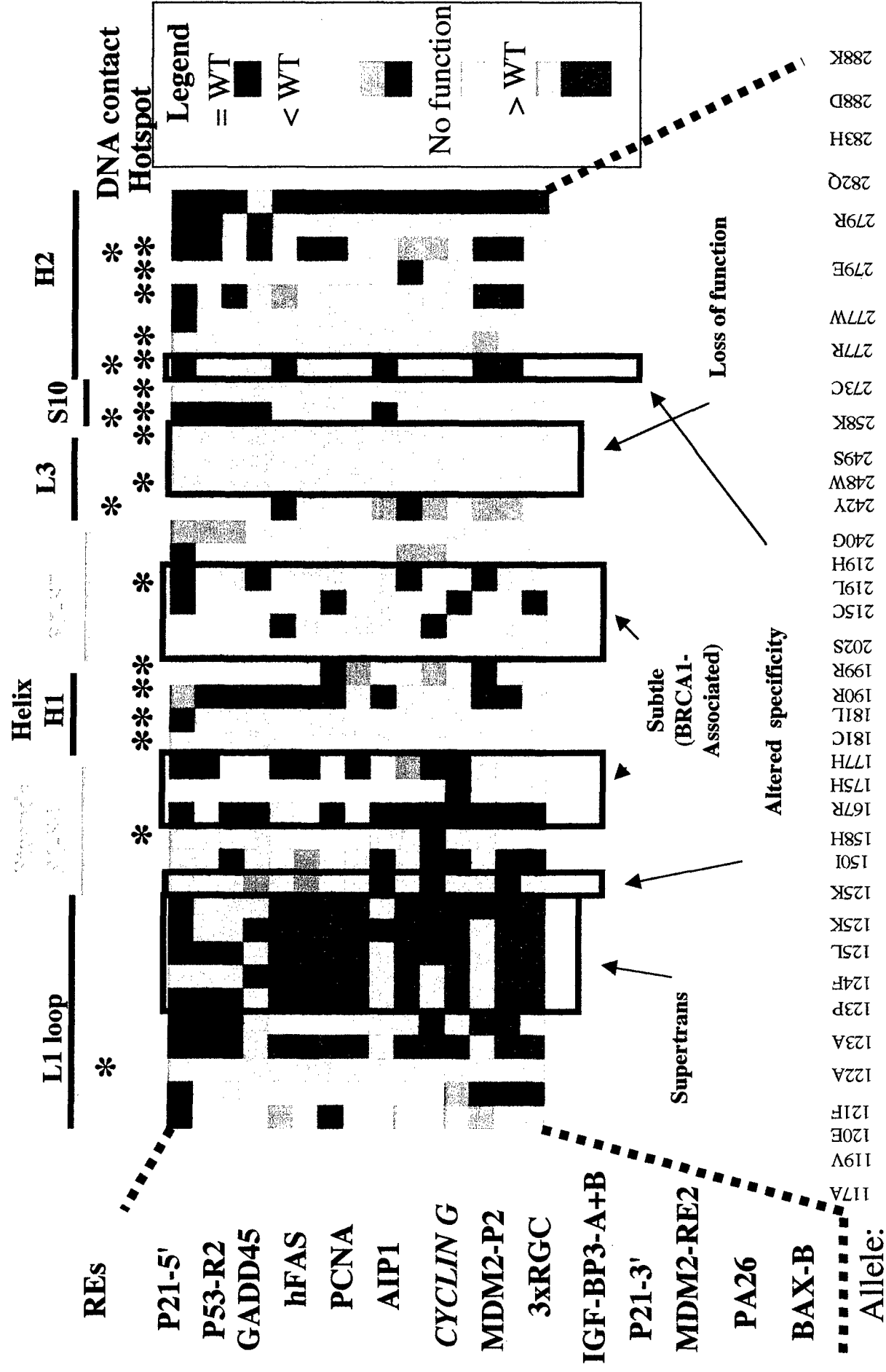| Galactose (%)	0	0.002	0.008	0.032	0.12
	(red)				(white)

p53 protein →

APPENDIX 3

Figure 1. p53 Transactivation Matrix.

The transactivation capacity of forty p53 mutant alleles relative to wt p53 is presented for

the fourteen p53 response elements listed in the left column. A black square indicates that

the mutant allele is not distinguishable from wt p53. Yellow to red color indicates a

progressive reduction in detectable transactivation. Light to dark green color indicates

different degrees of enhanced transactivation capacity. Pink indicates loss-of function.

The majority of p53 mutants in this panel retains partial transactivation function. The

mutant alleles (shown in the bottom) are ordered on the primary structure whose

topological domain are indicated at the top of the figure. Strands S1-S4 and S5-S7 are not

facing the DNA according to the crystal structure of p53 bound to DNA (21). DNA

contact sites, hotspots and functional classes are indicated. A group of mutants in the L1

loop showed increased activity with all response elements. These supertrans mutations

(22) do not occur in tumors but are capable of reactivating tumor mutations in cis

suggesting that conformational changes in the L1 loop possibly inducible by small

molecules could restore transactivation activity to p53 mutant protein expressed in
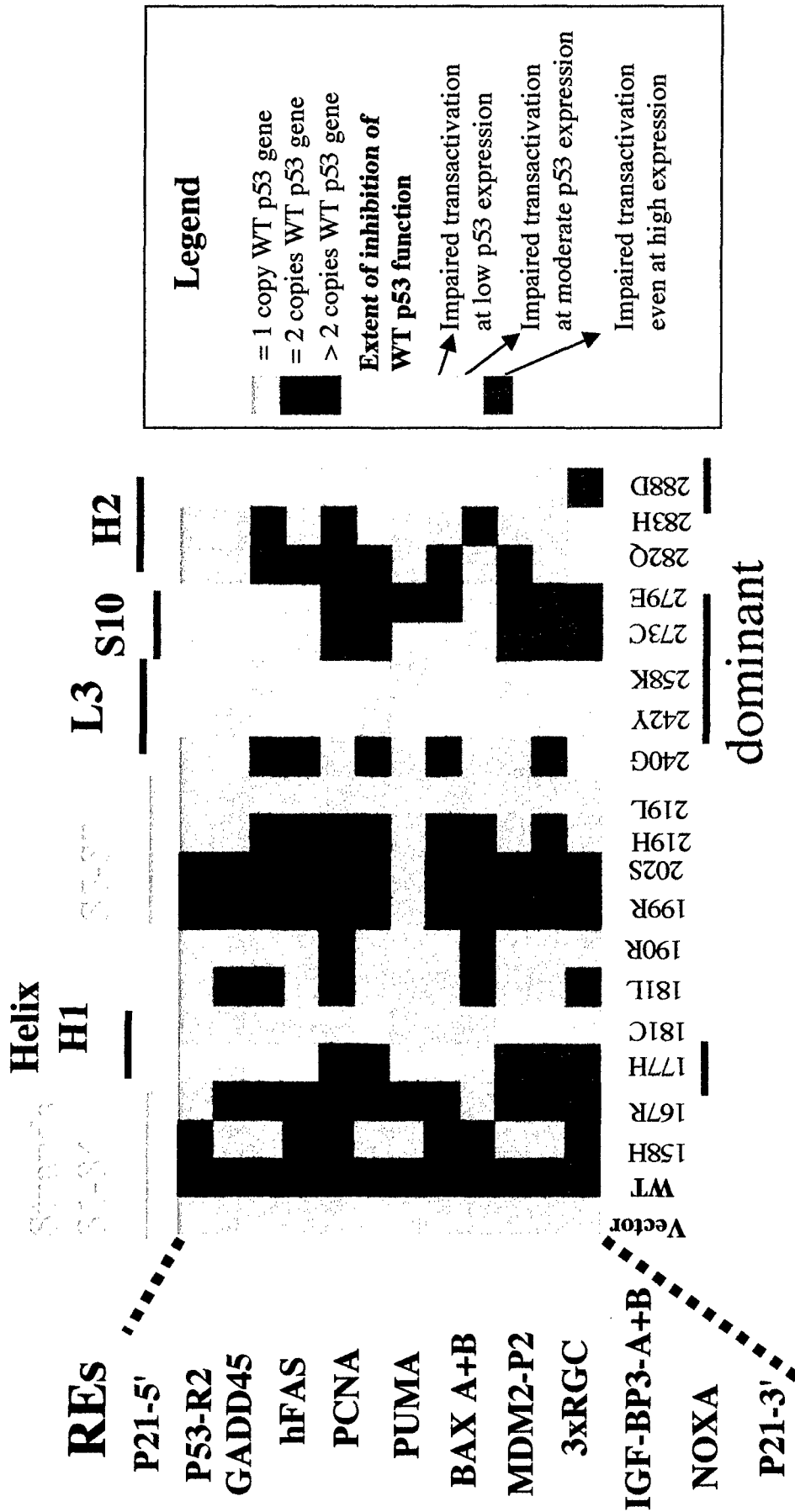
tumors (23).

Figure 2. p53 Dominance Matrix: co-expression of mutant and wt p53.

Eighteen of the forty mutants analyzed in Figure 1 were also evaluated when expressed at

equal levels with wild type p53. Interestingly the transactivation assay at low/variable

p53 expression under the *GAL* promoter was sensitive to gene dosage. Some of the p53

mutants associated with familial breast cancer that had revealed subtle defects in the

transactivation assays were completely recessive, while others led to a reduction of p53-

dosage phenotype. Different degrees of dominance were observed among non-functional

p53 mutants with mild to severe inhibition of wt p53 activity.

A. Functional p53 Transactivation Matrix

Legend
= WT
< WT
No function
> WT

DNA contact
Hotspot

L1 loop   Helix H1   L3   S10   H2

REs
P21-5'
P53-R2
GADD45
hFAS
PCNA
AIP1
CYCLIN G
MDM2-P2
3xRGC
IGF-BP3-A+B
P21-3'
MDM2-RE2
PA26
BAX-B

Allele: 117A, 119V, 120E, 121F, 122A, 123A, 123P, 124F, 125L, 125K, 125K, 150I, 158H, 167R, 175H, 177H, 181C, 181L, 190R, 199R, 202S, 215C, 219L, 219H, 240G, 242Y, 248W, 249S, 258K, 273C, 277R, 277W, 279E, 279R, 282Q, 283H, 288D, 288K

Supertrans

Subtle (BRCA1-Associated)

Altered specificity

Loss of function

B. p53 Dominance Matrix: Co-expression of mutant and WT p53

Legend

= 1 copy WT p53 gene
= 2 copies WT p53 gene
> 2 copies WT p53 gene

Extent of inhibition of WT p53 function

Impaired transactivation at low p53 expression

Impaired transactivation at moderate p53 expression

Impaired transactivation even at high expression
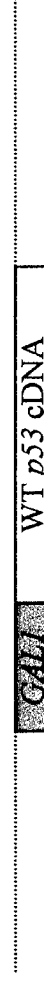
APPENDIX 4

**Figure 1. Development of a p53 *mutant functionality database*. A. Construction of any
missense p53 mutant can be accomplished rapidly with the *delitto perfetto in vivo*
mutagenesis system** (13). The *GAL1:p53* expression cassette is cloned into a yeast
chromosomal locus to obtain a p53-host strain. A number of isogenic derivatives of this
strain are created that have a CORE cassette (containing a COunter-selectable gene and a
REporter gene) inserted at different sites (amino acid 250 in the example). By placing a
CORE cassette at 200 nucleotides intervals, the entire p53 can be subjected to
mutagenesis. Introduction of oligonucleotides that surround the CORE allows for
creation of specific mutants (R273H in the example) or the generation of many different
mutants in a small region if degenerate oligonucleotides are used. Only DNA sequencing
of the region surrounding the replacement site is needed to confirm the nature of the
induced mutation(s) (13).

B. Transactivation assays using the Functional Array of p53 response elements. Over 20
isogenic yeast strains that differ only in the sequence of a p53 response element upstream
of the p53-reporter gene *ADE2* (a luciferase reporter gene can also be used to quantify
transactivation based on our unpublished observations) are mated with the p53-host strain
expressing a given p53 mutant. Diploids are selected on suitable media and then tested
with the phenotypic transactivation assay to determine the functional fingerprint of the
p53 allele being tested. The analysis of the dominance potential of p53 mutants can be
performed when the p53-host strain is transformed with a second *GAL1*:WT p53
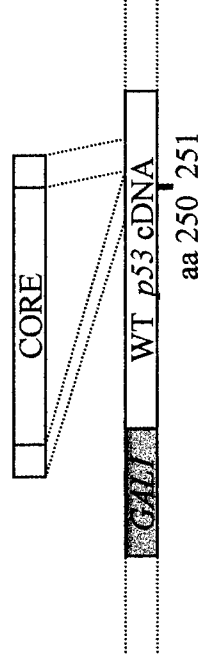expression cassette.

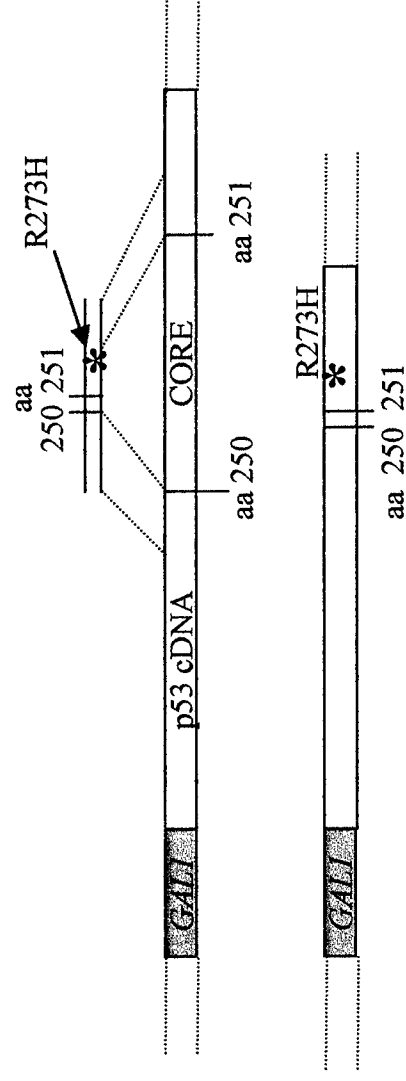*Delitto perfetto* strategy for *in vivo* site-directed mutagenesis of p53

1) p53 cDNA under a *GAL1* promoter is cloned into a yeast chromosomal locus by homologous recombination
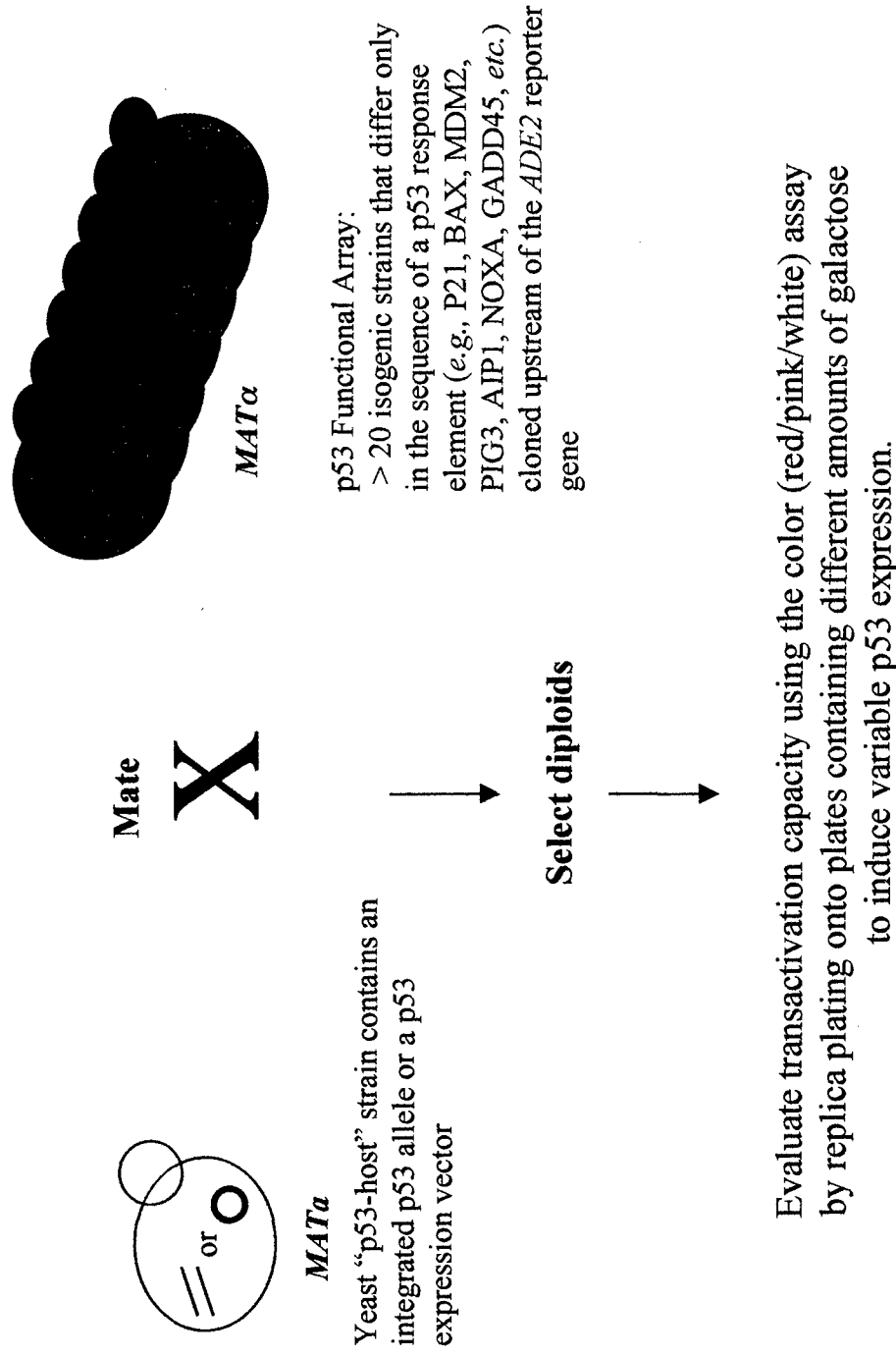

2) A CORE cassette (consisting of a Counterselectable and a Reporter marker) is integrated into the p53 cDNA at a specific position (*e.g.*, aa 250) without deleting any *p53* sequence

3) The CORE cassette is replaced by oligonucleotides containing a desired mutation or by degenerate oligonucleotides for random mutagenesis (in the example, the CORE is integrated between aa 250 and 251 and a mutation at codon 273 is created by oligonucleotide replacement)

Strategy for the determination of p53 allele functional fingerprints using the p53 *Functional Array*

MATa

Yeast "p53-host" strain contains an
integrated p53 allele or a p53
expression vector

Mate

X

MATα

p53 Functional Array:
> 20 isogenic strains that differ only
in the sequence of a p53 response
element (*e.g.*, P21, BAX, MDM2,
PIG3, AIP1, NOXA, GADD45, *etc.*)
cloned upstream of the *ADE2* reporter
gene

Select diploids

Evaluate transactivation capacity using the color (red/pink/white) assay
by replica plating onto plates containing different amounts of galactose
to induce variable p53 expression.

TABLE OF CONTENTS

INTRODUCTION

Multiple perturbations of the complex network of signaling pathways that define the

precise role of a cell in its tissue microenvironment and that regulate stress responses are

accumulated through genetic and epigenetic changes during processes of transformation

that lead to cancer (1). The tumor suppressor gene p53 plays a major role in cellular

response to various environmental stresses (2). The p53 protein is a sequence-specific

transcription factor that can lead to transactivation and repression of over 100 genes,

many of which are involved in apoptotic or cell cycle arrest responses. The induction of

p53 responses can lead to different biological effects according to the cell type or the

activating stimuli. For example, temporary G1 or G2 cell cycle arrest, premature

senescence and programmed cell death can all be induced (or maintained) by p53 (3).

The mechanisms of how the choice between these pathways is brought about are still

elusive, although several possibilities have been proposed. Differential regulation of

downstream genes, both in terms of the extent and the kinetics of transactivation and

repression, is likely to be an important factor for dictating the specificity of p53

biological responses. Functional alterations in the p53 pathway likely occur in nearly all

human cancers. In almost half the human malignancies, there is a mutation in the p53

gene itself (4, 5). Interestingly, ~80% of p53 mutations are missense changes that lead to

single amino acid substitutions, a feature that distinguishes p53 from other tumor

suppressor genes (*e.g., APC, NF1, BRCA1*) (6). The incidence of p53 mutations and the

types of mutations can vary among tumors in different tissues or populations (4) and the

exposure to some environmental agents has been associated with the features of p53

mutation spectra in tumors (7). About 1300 different amino acid changes at p53 have

been reported in tumors and a significant fraction of these do not lead to loss of function,

but can retain partial functions or exhibit altered biological activities. Several recent

reports support the view that certain *p53* mutant alleles can retain partial function. Some

mutations are associated with partial transactivation and are capable of inducing G1

arrest, but not apoptosis (8). Furthermore, a recent analysis of 77 different p53 mutant

proteins from tumors revealed that >15% could still activate a yeast promoter containing

a *p21* RE, but not the *Bax* or *PIG3* RE (9), which presumably is important in tumor

development. A few *p53* mutant alleles appear to have acquired functions that may

provide a selective advantage to tumor cells, such as the up-regulation of growth

promoting genes (*e.g., Myc, MDR, VEGF*) (10). Finally, *p53* mutant alleles that appear

normal for transactivation, growth suppression and apoptosis when ectopically expressed

at high levels in a tumor cell line, have been detected in breast and ovarian tumors

associated with *BRCA1* defects (11). Hence, detailed functional analysis of multiple

tumor *p53* alleles is expected to provide valuable information in predicting tumor state,

including aggressiveness and responsiveness to therapy.

We developed functional assays based in the yeast *Saccharomyces cerevisiae* that

provide for functional classification of p53 alleles using quantitative gene reporter assays

in a constant chromatin structure. The systems rely on *rheostatable* regulation of p53

expression and determine the transactivation capacity of p53 mutants relative to wild-

type p53 or the dominance potential of mutants when heterozygous with wild-type p53

using a large spectrum of p53 Response Elements (REs). The yeast functional assay

offers a practical means to develop a *p53 mutant functionality database* that collects the

functional fingerprints for all the p53 mutants relevant to cancer to be linked with the

IARC p53 mutation database. This information will become valuable in understanding

the correlation between p53 functional status and clinical outcome, thus providing

directions for effective patient management in the clinical setting.

RESULTS (BODY)

A detailed description of the experimental approach, results and the discussion of the implications of this work are presented in the appended publication (see Appendix 1). A general scheme of p53-regulated transcriptional targets and their functions is presented in Appendix 2, Figure 1. Figure 2 shows the distribution of p53 mutations reported in tumors along with the topological, functional and evolutionary conserved domains of the protein. Figures 3 A-C describe the yeast-based p53 functional assay we developed drawing in part from previously published works (12). Examples of the functional fingerprinting (both transactivation and dominance) of p53 mutant alleles reported in familial and sporadic breast cancers are presented in Appendix 3. A general strategy for the construction of any p53 mutant allele of choice based on our recently developed *delitto perfetto in vivo* mutagenesis system (13) is presented in Appendix 4, Figure 1A. A high-throughput experimental scheme for determining the transactivation capacity of any p53 mutants at many promoter elements is also presented in Figure 1B.

The following is a summary of the results described in the appended publication (Appendix 1).

1) **Development of a panel of isogenic yeast strains containing p53 responsive promoters** (pages 2-3 of Appendix 1).

26 different p53 REs were selected from p53-regulated promoters of genes involved in different biological pathways including cell cycle arrest, apoptosis (both mitochondrial and receptor pathway), DNA repair and regulation of p53 stability/activity. Simple REs that provides for a p53 tetramer binding site were integrated in the same chromatin locus to regulate the expression of the ADE2 (red/white) reporter gene.

2) **Development of a tightly regulated p53 expression cassette in yeast based on a rheostatable *GAL1,10* promoter** (page 4 and FIG.2 of Appendix 1).

The *GAL* promoter is repressed on glucose, while on raffinose a derepressed, higher basal transcriptional state is achieved (14). We observed that the p53 level on glucose is about 15 times lower than on raffinose based on densitometric analysis of Western blots. Varying the amounts of galactose inducer added to the raffinose medium lead to a gradual induction of the promoter over a wide range. The induction of p53 expression was approximately linear over the galactose range of concentration from 0% to 0.12%.

3) **Rheostatable expression of wild-type *p53* revealed a broad range of transactivation capacities with the different REs** (pages 4-6 and FIG. 3-4 of Appendix 1).

Transactivation of *ADE2* by p53 results in pink and white colonies, depending on the extent of induction of the *ADE2* reporter.

The relative ability of p53 to transactivate the reporter gene at various REs was examined by using different levels of galactose inducer and ranking the amount of galactose required for turning colonies from red to pink (= weak transactivation) and to white (= strong transactivation). The 26 *yAFM*-REs could be ranked for ability to be induced by different amounts of p53 using this phenotypic color assay. Surprisingly, there was as much as a 1000-fold difference in transactivation. Our results suggest that intrinsic DNA binding affinity, as well as p53 protein levels, are important contributors to p53-induced differential transactivation. We found that p53 had weak activity towards the apoptotic REs of the mitochodrial pathway of programmed cell death.

4) Transactivation capacity measured by color assay corresponds to *ADE2* mRNA levels (pages 6-7 and FIG. 6 of Appendix 1).

The red/pink/white phenotypic assay for assessing p53 gene-specific transactivation capacity and the impact of different levels of expression is simple and highly reproducible. However, the mechanism of red pigment accumulation and metabolism by the *ADE2* gene product to result in white colonies is unclear and might be affected by processes that are independent of p53.

We addressed the relationship between colony color and *ADE2* transcription using a quantitative PCR approach in real-time. These experiments revealed that there were comparable levels of *ADE2* expression from various REs in the absence of p53 protein and that the levels of mRNA induced by p53 at various REs reflected colony color, indicating a good correlation between the phenotypic assay and transactivation of the *ADE2* gene.

5) Statistical predictions of binding probability of the REs based on nucleotide usage do not correlate with the functional rank (pages 8 and 11 of Appendix 1).

Neither the number of non-consensus bases in the REs sequence nor statistical methods based on nucleotide usage [Heterology index (15) as well as MH-Algorithm (16)] predicted the wide variations in transactivation capacity we observed among the panel of p53 REs.

6) The CATG sequence at the center of a p53-dimer binding site greatly affects RE activity (pages 8 and 11 of Appendix 1).

We hypothesize that sequence-dependent structural features of the DNA greatly affect p53 activity in our *in vivo* system, as previously observed *in vitro* with purified p53 DNA binding domain and naked DNA RE (17). It is possible that upon interaction with DNA

RE sequences the p53-DNA complex undergoes conformational changes that significantly contribute to binding affinity and activity. Consistent with this we noticed that the four strongest REs have the 5'-CATG sequence at the junction between p53 monomer binding sites in both dimer sequences. Previous in vitro studies showed that the flexibility of the CATG sequence facilitates axial bending of the DNA upon interaction with p53 protein. The construction of artificial p53 REs with defined changes at the junction between monomer binding sites confirmed the important role of the CATG sequence in transactivation capacity.

7) **Subtle changes in transactivation capacity are revealed in *p53* mutations associated with familial breast cancer** (page 8 and Table 2 of Appendix 1).
The incidence of p53 mutations in familial breast cancer associated with germline BRCA1/2 mutations is nearly 70% as compared to around 30% for sporadic breast cancer. Moreover, the spectrum of mutations appears to be different(18). Interestingly, a subset of BRCA1-associated p53 mutant alleles appeared wild type in mammalian functional assays (11) and 4 of the mutants, T150I, G199R, R202S, and S215C, were also wild type in a yeast transactivation assay at high p53 expression (9). We determined the transactivation capacity of these four alleles relative to wt p53 using the rheostatable *GAL* promoter system for p53 expression. Only under conditions of low expression we were able to detect subtle changes in transactivation comprising both enhanced and reduced activity for several REs

8) Functional fingerprinting of p53 mutant alleles reported in sporadic and familial breast cancer (Figure 1 and 2 of Appendix 3).

A larger panel of p53 mutant alleles and REs was analyzed with the yeast system in order to develop functional fingerprints. Both transactivation capacity and dominance potential were determined.

Forty p53 mutations were tested including DNA contact mutants, mutation hotspots in breast and other cancer types, mutations preferentially associated with familial breast cancer, and novel alleles in the L1 loop of the DNA binding domain. Figure 1 in Appendix 3 contains a graphical representation of the transactivation capacity of these alleles relative to wt p53. The yeast functional assay at variable p53 expression and with many p53 response elements revealed both subtle and dramatic changes in transactivation including increased activity compared to wt p53. A significant fraction of p53 mutants retain partial function in this analysis, including some tumor hotspots. Interestingly, the group of p53 alleles preferentially associated with familial breast cancer showed a unique functional fingerprint characterized by subtle increase and decrease in activity with several REs.

The analysis of the dominance potential (Figure 2 in Appendix 3) of p53 mutants when expressed at equal low/variable levels with wt p53 was sensitive to the p53 gene dosage since it distinguished the presence of one versus two wt p53 alleles. Different degrees of dominance were observed with the tumor alleles. Consistent with the transactivation results the p53 mutants associated with breast cancer were not dominant although some caused a reduction of gene dosage phenotype.

KEY RESEARCH ACCOMPLISHMENTS

1) **Development of a panel of isogenic yeast strains containing p53 responsive promoters and of a rheostatable *GAL1,10* promoter system providing for tight regulation of p53 expression.**

2) **Determination of the intrinsic transactivation capacity of wild type p53 towards its many response elements (REs) using an *in vivo* system with constant chromatin structure.** A broad range of transactivation capacity was detected with low activity particularly for apoptotic REs of the mitochondrial pathway.

3) **Poor correlation between *in vivo* transactivation capacity and predicted binding probabilities based on statistical analyses.** Available algorithms estimating binding probabilities of p53 to its many REs did not predict the result of the functional assay. The difference in intrinsic transactivation capacity are likely to be influenced by mutually induced conformational changes of the DNA target site and the p53 protein upon recognition and interaction with a RE.

4) **Identification of subtle changes in transactivation capacity comprising both reduced and enhanced activities in a group of rare *p53* mutations associated with familial breast cancer, previously classified as wild type.**

5) **Use of the yeast-based assays for functional fingerprinting of p53 mutant alleles.** A significant fraction of p53 mutant alleles associated with cancer retain partial transactivation. Loss-of-function alleles exhibited different degrees of dominance over wt p53 when heterozygous.

6) **Development of the concept of** *the p53 mutants functionality database.* The p53

functional assays developed in yeast provide means to develop a *functionality*

database of all tumor p53 mutations associated with breast and other cancers with

implications to ascertain the correlation between p53 functional status and tumor

aggressiveness and responsiveness to therapy.

REPORTABLE OUTCOMES

The results of our work supported by the DOD Concept Award have been presented at

international meetings and in the publications listed below:

<u>presentation at meetings</u>

1) A sensitive *in vivo* system determines the transactivation specificity and selectivity of

human p53 alleles at many promoter elements. -Inga, Storici, Bouma, Darden, and

Resnick, poster presentation-

93rd Annual Meeting, American Association for Cancer Research, April 6-10, 2002, San

Francisco, CA, USA

2) Functional fingerprints of human p53 mutants using quantitative yeast-based

transactivation assays reveal specific changes in p53 activity that may be relevant to

tumor biology. -Inga, Storici, Darden, and Resnick, oral presentation-

2002 Yeast Genetics and Molecular Biology Meeting, July 30-August 4, 2002, Madison,

WI, USA

3) Functional fingerprints of human p53 mutants using quantitative yeast-based

transactivation assays reveal specific changes in p53 activity that are relevant to tumor

biology. -Inga, Storici, Bouma, Liu, King, Monti, Darden, Fronza, and Resnick, poster

presentation-

Era of Hope Department of Defense Breast Cancer Research Program Meeting,

September 25-28, 2002, Orlando, FL, USA

publications

1) A. Inga, F. Storici, T. A. Darden and M. A. Resnick (2002). Differential

transactivation by the p53 transcription factor is highly dependent on p53 level and

promoter target sequence. MCB, in press -see Appendix 1.

2) A. Inga, P. Monti, B. Bouma, G. Fronza, and M.A. Resnick. Functional fingerprinting

of tumor p53 alleles using quantitative yeast-based assays. -in preparation-

CONCLUSIONS AND FUTURE DIRECTIONS.

We developed new tools providing for the functional profiling of p53 alleles reported in

breast and other cancers both in terms of transactivation capacity and dominance over wt

p53. The results presented in Appendix 3 together with other unpublished observations

(Storici *et al.*, manuscript in preparation) strongly suggest that many p53 mutations

associated with cancer are likely to retain partial transactivation function *in vivo*.

Although several classification methods for p53 mutants have been attempted our

findings indicate that it is not presently possible to predict *a priori* the behaviour of a

mutant p53 protein. The yeast functional assays provide an important contribution to the

functional classification of p53 alleles. It appears that the combination of low and

variable expression of p53 alleles and the assessment of the transactivation capacity using many p53 response elements provides a greater sensitivity for the functional classification of p53 mutants than the standard yeast assays using high constitutive p53 expression. When combined with the efficient method for constructing p53 alleles using *in vivo* mutagenesis (13) (see Appendix 4, Figure 1A) and with the rapid screening system depicted in Figure 1B of Appendix 4, these tools allow for rapid development of p53 functional fingerprints.

Hence, the yeast functional assay offers a practical means to develop a *p53 mutant functionality database* that enables the functional fingerprints for all the p53 mutants relevant to cancer to be linked with the IARC p53 mutation database. Particularly in sporadic breast cancer, published and ongoing studies focus on the correlation between p53 status and clinical outcome. However, the functionality of the tumor p53 alleles has never been carefully considered in these analyses which yielded contrasting results. Recent reports highlighting the complex functional interactions between Estrogen Receptor, BRCA1 and p53 (19) further strengthen the value of detailed functional analyses of p53 mutant alleles reported in breast cancer. We propose that a *p53 mutant functionality database* will become valuable in understanding the correlation between p53 functional status and tumor aggressiveness and responsiveness to therapy, and may provide directions for effective patient management in the clinical setting.

The financial support from the Department of Defense Breast Cancer Research Program has been instrumental in the development of these yeast-based functional-assay systems. We hope that future support would allow us to focus our investigation on elucidating the prognostic value of p53 functional status in breast cancer.

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